STUDY THE POTENTIAL OF BIOTIC AND ABIOTIC COMPOUNDS AS ALTERNATIVE TO ANTI FUNGAL ANTIBIOTIC AGAINST MDR *Candida spp.*

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE
IN
MICROBIOLOGY

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June 2025

MSME
MICRO, SMALL & MEDIUM ENTERPRISES
सूक्ष्म, लघु एवं मध्यम उद्यम

UDYAM-WB-08-0047015
LIC NO-027022003722

REF NO: BIOPIONEER/CER/2024-25/__

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hands-on skill development program titled

"Study the potential of Biotic and Abiotic compounds As Alternative To Antifungal Antibiotic Against MDR Candida Species."

from 01.03.2025 to 31.03.2025 AT AM Educare Research Laboratory for the fulfilment of part of curriculum

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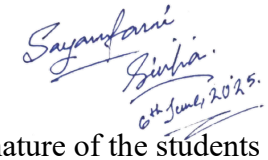

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This is to certify that the project report titled " STUDY THE POTENTIAL OF BIOTIC AND ABIOTIC COMPOUNDS AS ALTERNATIVE TO ANTI FUNGAL ANTIBIOTIC AGAINST MDR *Candida spp*.", submitted by Ms. Sayantani Sinha (Registration No. 230010243095 Enrolment No. 2322211001024) to the Department of Microbiology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by her for the partial fulfillment of the requirements for the Master of Science degree.

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ACKNOWLEDGEMENT

I want to show my deepest appreciation to my supervisor **Dr. Anirban Mukherjee** because of their continuous support and guidance throughout this project. The direction of my work together with its quality greatly improved through the expert guidance and patient feedback from my supervisor.

The faculty at **Sister Nivedita University** built an academic environment which motivated me to explore through critical thinking. Special thanks to Professors. My research received valuable insights from Head of Department, Microbiology **Professor Fatema Calcuttawala** and Assistant Director of Department **Dr. Rajat Pal** that improved its quality.

My deepest thanks go to my peers and friends who made my academic journey better through their friendship and stimulating discussions and motivation. Their backing transformed the whole academic experience into a rewarding and pleasurable adventure.

I express gratitude to everyone who contributed to the successful project completion whether explicitly named or not. Your attendance and backing facilitated the successful achievement of this accomplishment.

Sayantani Sinha

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List of Abbreviations

Abbreviation	Full Form
MDR	Multidrug-Resistant
SDA	Sabouraud Dextrose Agar
TLC	Thin Layer Chromatography
MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
PAGE	Polyacrylamide Gel Electrophoresis
UV	Ultraviolet
NiNP	Nickel Nanoparticles
ZOI	Zone of Inhibition
DMSO	Dimethyl Sulfoxide
TE Buffer	Tris-EDTA Buffer
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
APS	Ammonium Persulfate
TEMED	Tetramethylethylenediamine
NB	Nutrient Broth
SDB	Sabouraud Dextrose Broth

Abstract

The increasing emergence of multidrug-resistant (MDR) *Candida* species poses a critical challenge to public health, particularly within hospital settings and among immunocompromised patients. Existing antifungal therapies, including azoles and polyenes, have shown diminishing efficacy due to adaptive resistance mechanisms such as biofilm formation, efflux pump activity, and genetic mutations (Espinel-Ingroff et al. [8]). This project investigates the antifungal potential of both biotic and abiotic agents as alternative strategies to combat MDR *Candida spp.*

Biotic agents studied include methanolic curcumin extract from turmeric, a siderophore derived from fish gut microbiota, a probiotic culture of *Lactobacillus rhamnosus*, and a biopolymer synthesized from polyethylene glycol and citric acid. The abiotic agent tested was a nickel nanoparticle synthesized using curcumin as a reducing and stabilizing agent. The project began with soil-based isolation of MDR *Candida* strains, confirmed through antifungal drug susceptibility testing. A series of assays, including cup-plate, MIC, MTT, anti-biofilm, and biofilm eradication assays, were used to evaluate the inhibitory effects of these agents.

The findings revealed that nickel nanoparticles and curcumin extract exhibited the highest zones of inhibition in cup-plate assays, while fish gut siderophores and the biopolymer showed moderate to no visible inhibition (Rai et al. [2]; Sharma et al. [1]). Further investigations demonstrated that several of these compounds impaired biofilm formation and eradicated pre-formed biofilms, with nickel nanoparticles producing notable anti-biofilm activity. MTT assays provided insight into reduced cell viability, and MIC values indicated the minimum concentrations required for inhibition. DNA damage studies using PCR and gel electrophoresis showed genotoxic effects in treated *Candida* cells, especially with nickel nanoparticles and curcumin. Additionally, SDS-PAGE and protein stress profiling revealed altered protein expression patterns under compound-induced stress, supported by bioinformatics docking simulations of ligand-protein interactions.

Overall, the study demonstrates that natural and synthetic compounds—particularly curcumin and nickel nanoparticles—have significant potential as antifungal agents. These alternatives not only inhibit fungal growth but also affect biofilm structure, viability, and genetic integrity, offering a promising avenue for future antifungal drug development against resistant *Candida* species.

Keywords:

Multi drug resistant, Curcumin, Nickel Nanoparticle, Probiotic, Biopolymer, Fish Gut Microbiota, Siderophore, Biofilm, Antifungal, Minimum Inhibitory Concentration (MIC), PCR, SDS PAGE.

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Introduction

Candida infections were once manageable illnesses, mostly seen in individuals with compromised immune systems. In contrast, recent hospital outbreaks involving *Candida auris*, *Candida glabrata*, and *Candida krusei* have become increasingly challenging to treat because of the rise in multidrug resistance (MDR).

Traditional antifungal drugs including azoles and echinocandins and polyenes have become ineffective against *Candida* strains because they developed resistance mechanisms through efflux pump overexpression enzyme mutations biofilm production and stress adaptation (Vila et al. [3]). The development of treatment-resistant fungi has created an immediate need for new medical approaches with specific emphasis on biotic and abiotic substances.

Biotic compounds obtained from plants and bacteria and fungi and animals demonstrate potent antifungal properties (Singh et al. [4]). Eugenol and thymol and carvacrol substances from thyme and oregano and cinnamon disrupt fungal membranes and prevent ergosterol synthesis in fungi. AMPs including defensins and cathelicidins break fungal membranes and direct immune response activation. The growth of *Candida* along with biofilm formation receives inhibition from microbial compounds produced by Bacillus and Pseudomonas species and the probiotics Lactobacillus and Bifidobacterium.

Metals in nanoparticle form (such as silver and zinc oxide) together with ionic liquids represent emerging antifungal solutions (Bai et al. [10]; Rai et al. [2]). The anti-fungal properties of these substances result from oxidative stress and membrane damage effects while nanoparticles show particular promise because they are difficult for fungi to develop resistance against. Medical research focuses on developing synthetic polymers and hydrogels containing embedded antifungal properties.

The therapeutic power of antifungal drugs increases when pairing them with both biological and non-biological compounds while minimizing toxicity and improving penetration into biofilms. Combining antifungal compounds produces treatment effects that help minimize the amount of medication needed and extend the time until drug resistance develops.

The research investigates selected abiotic and biotic compounds when used against MDR *Candida* while studying their effectiveness in planktonic and biofilm states to determine mechanisms of action and treatment compatibility. The research aims to develop safer antifungal treatments for MDR *Candida* infections which represent a critical international health problem.

Our study investigates *Candida* species which represents a fungus notoriously difficult to treat with available drugs. The current antifungal medicines lack sufficient power to eliminate *Candida* thus creating major difficulties for medical treatment. The environmental fungus exists throughout the environment where it spreads to infect human bodies. The research investigates whether five natural and abiotic factors show promise against *Candida*.

The research process starts with isolating *Candida* from environmental soil. We perform disk diffusion tests with different available antifungal drugs to determine fungal resistance levels of cultured cells. The test confirms that our biological strain possesses multidrug resistance characteristics.

We proceed with preparing our five experimental factors after finishing the initial characterization process. The extraction and analysis of curcumin as a compound from turmeric occurs through Thin Layer Chromatography (TLC) methods. The analytical method of Thin Layer Chromatography (TLC) separates mixtures by identifying and quantifying their components while using a thin layer adsorbent plate. A small sample amount is applied to an adsorbent material layer that coats the plate surface where the material is typically silica gel or alumina or cellulose. The plate containing the sample travels through the solvent because of capillary action. The sample components separate into distinct spots at different heights on the plate because different chemical properties control how quickly each component moves with the solvent. Nanobiotechnology combines nanotechnology and biology as an interdisciplinary discipline for molecular and atomic scale tool and material development. Nanoscale devices and systems develop through this field for use in medical applications as well as agricultural and environmental scientific domains. The research area focuses on enhancing diagnostics along with drug delivery methods and bioengineering applications using nanotechnology principles. The nano-biotechnology methods produce Nickel nanoparticles which function as an abiotic agent. A sodium chloride-based extraction method helps us prepare a siderophore from Tilapia fish gut which we obtain from the gut tissue. The study incorporates

Lactobacillus rhamnosus obtained from a commercial pro biotic capsule as one of its experimental components. The last stage of our research involves bio-polymer synthesis for additional evaluation of biotic compounds.

A cup-plate assay helps us determine the effectiveness of these factors against *Candida* growth. We produce agar plates followed by the creation of five wells before adding each factor to a different well. The inhibition zones surrounding the wells during incubation serve as an initial assessment of anti-fungal potency against existing treatments for each factor.

A 96-well plate assay enables us to conduct many experiments simultaneously. Our factors demonstrate their ability to break down biofilms which *Candida* cells along with other microorganisms use as protective layers against environmental dangers. The research investigates if these factors can stop biofilm formation during both treatment and prevention stages.

The study requires information about *Candida* cell survival rates after treatment procedures. An MTT assay helps us determine cell survival rates while absorbance measurement leads to visualization of fungal cell populations post-factor exposure. The MIC assay determines agent potency by testing successive factor dilutions to identify the lowest effective killing concentration against *Candida*.

The investigation extends past cell death assessment to determine if the factors inflict molecular damage to the fungus. The research team extracts *Candida* cell genomic DNA followed by gel electrophoresis analysis to monitor DNA structural or density variations. Our evaluation includes an assessment of whether these factors harm both DNA and proteins in fungal cells to better understand their assault mechanisms.

Our intention is to unite these various observations for determining if our selected natural and abiotic elements surpass current antifungal drugs in their ability to fight *Candida*. Computational Drug Designing implements computer-based tools alongside simulations for the creation and enhancement of new drug *Candida*tes. Computational Drug Designing uses mathematical modeling to simulate drug-target interactions which enables both faster drug development and shorter timelines and decreased experimental spending. We aim to contribute to the development of improved methods that will help manage fungal infections which have become resistant to multiple drugs.

Review of Literature

In recent years, *Candida* species have evolved from being relatively manageable fungal pathogens to major challenges in clinical environments, especially among immunocompromised individuals. The emergence of multidrug-resistant (MDR) strains such as *Candida auris*, *C. tropicalis*, and *C. krusei* has significantly weakened the effectiveness of commonly used antifungal drugs. Traditional treatments like azoles and echinocandins now face resistance due to mechanisms such as efflux pump activity, biofilm development, and genetic changes in the fungal cells. As drug failure becomes more frequent, the need for novel therapeutic strategies has grown more urgent.

One alternative approach gaining attention involves the use of naturally derived biotic compounds. Curcumin, a yellow pigment extracted from turmeric, has demonstrated promising antifungal properties. It disrupts fungal cell membranes and interferes with vital metabolic pathways, including those involved in cell wall integrity. Despite these benefits, curcumin's medical application is limited by its poor solubility and low systemic absorption. To overcome this, researchers are exploring its use in combination with other delivery systems, particularly nanotechnology-based carriers.

Another biotic strategy focuses on microbial metabolites. Siderophores, small iron-binding molecules secreted by certain bacteria, play a crucial role in microbial survival under iron-limited conditions. When used in antifungal applications, siderophores can reduce fungal access to iron, thereby limiting growth. Probiotic strains such as *Lactobacillus rhamnosus* have been investigated for their ability to produce these compounds and indirectly inhibit pathogenic fungi. Similarly, fish gut microbiota have shown potential as an underexplored source of antifungal agents.

Abiotic solutions are also being examined, particularly the use of metal nanoparticles. Among these, nickel nanoparticles (NiNPs) synthesized using plant-based reducing agents have shown significant antifungal potential.

These particles can damage fungal cells by disrupting their membranes and inducing oxidative stress. What sets them apart is the difficulty fungi have in developing resistance against them due to their multifaceted mode of action. The synthesis process, which involves green chemistry principles, also adds an environmentally friendly dimension to their use.

In addition, synthetic biopolymers offer interesting possibilities. Though they may not always possess direct antifungal effects, they serve as excellent drug carriers due to their biocompatibility and ability to deliver therapeutic compounds in a controlled manner. Polymers developed from naturally derived agents like citric acid and polyethylene glycol are being studied for this purpose, particularly when combined with active antifungal agents such as curcumin.

Altogether, these studies suggest a shift away from reliance on conventional antifungal medications toward more diverse and integrative methods. Whether through natural plant extracts, microbial products, or engineered nanomaterials, a multi-pronged approach appears increasingly necessary to manage and eventually overcome the challenge posed by MDR *Candida* strains.

Material and Chemical Instruments

1. Glassware and Tools

- Test Tubes
- Falcons
- Conicals
- Beaker
- Measuring Cylinder
- Glass Rods
- Capillary Tubes
- MCT Tubes
- Eppendorf
- Pipette (Bexco)
- Tips
- Petri Plates
- Spreader
- Inoculation Loop
- Spatula
- Spirit Lamp
- Syringe
- Ice Box
- Gloves (Microflex)
- Marker
- Sissors
- Tape
- Tissue Paper
- Rubber Bands
- Filter Paper
- Funnel

2. Chemicals

- n - hexane
- Methanol
- Chloroform
- Ethanol
- NaCl (Saline)
- Phenol
- Isoamyl Alcohol
- TE Buffer
- SDS
- TAE Buffer
- Saboraud dextrose
- Acetic Acid
- Bromophenol Blue
- ETBr
- Ammonium per Sulphate
- TEMED
- Tris HCl
- Acrylamide
- Acetone
- Glycerol
- Beta-marcapto Ethanol
- Coomassie Brilliant Blue (CBB)
- Gram's Crystal Violet
- Proteinase K
- RNAse
- EDTA

3. Anti-Fungal Drugs

- Nystatin
- Amphotericin
- Itraconazole
- Clotrimazole
- Fluconazole
- Miconazole
- Ketoconazole

4. Instruments

- Autoclave
- Laminar Air Flow
- Weighing Scale
- Spectrophotometer
- UV Light
- Microwave Oven
- Gel Electrophoresis Apparatus
- Centrifuge
- Dry Bath
- Vortex
- -4, 20 Fridge
- Incubator
- Magnetic Stirrer
- ELISA Reader
- PCR Machine
- SDS PAGE Gel apparatus

Isolation of *Candida* from Soil:

Methods:

1. Preparation of Sabouraud Dextrose Agar (SDA)

Sabouraud Dextrose Agar was prepared by dissolving glucose, peptone, and agar in distilled water within a sterile conical flask. This medium is suitable for the growth of fungi, including Candida species.

2. Sterilization of Media and Glassware

All glassware, including the prepared SDA medium, was sterilized using an autoclave. The sterilization was carried out at a pressure of 15 PSI to ensure complete elimination of contaminants.

3. Agar Plate Preparation

Once autoclaved, the hot SDA medium was carefully poured into sterile Petri dishes under a laminar airflow hood to prevent contamination. The plates were left to cool and solidify before use.

4. Serial Dilution of Soil Sample

To isolate fungal colonies from soil, a serial dilution technique was employed as follows:

- A 10-gram soil sample was measured and transferred into a sterile container.
- 90 mL of sterile distilled water was added to the soil.
- The mixture was shaken vigorously for 2 minutes to create a uniform soil suspension.

Step-by-step Dilution Process:

i. Four sterile test tubes were labeled as 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}.

ii. Each tube was pre-filled with 9 mL of sterile distilled water.

iii. 1 mL of the original soil suspension (considered the 10^{-1} dilution) was added to the 10^{-2} tube.

iv. The tube was gently but thoroughly mixed.

v. Next, 1 mL from the 10^{-2} tube was transferred into the 10^{-3} tube.

vi. Mixing was repeated to ensure even distribution.

vii. The dilution process continued sequentially to prepare the 10^{-4} and 10^{-5} tubes.

5. **Plating and Incubation**

From each dilution, 100 µL of the suspension was carefully pipetted onto separate SDA plates. The inoculum was spread evenly using a sterile glass spreader to allow isolated colony formation. All plates were incubated at room temperature for 48 hours, during which microbial growth was observed and monitored.

Multi drug resistant assay of *Candida*

1. A 250 mL volume of Sabouraud Dextrose Agar (SDA) was prepared by dissolving the required ingredients in a sterile conical flask.

2. The prepared SDA medium, along with all necessary glassware, was sterilized in an autoclave at 15 PSI to ensure complete microbial decontamination.

3. Following autoclaving, the sterilized materials were transferred to a laminar airflow chamber to maintain a sterile working environment.

4. The work surface inside the laminar hood was disinfected thoroughly to eliminate any potential contaminants.

5. The hot SDA medium was poured into pre-sterilized Petri dishes and allowed to cool and solidify under aseptic conditions.

6. After the agar had set, 100 µL of *Candida* cell suspension was drawn and added to a sterile liquid culture medium to prepare for plating.

7. Using a sterile glass spreader, the *Candida* suspension was evenly distributed across the surface of the solidified agar plate.

8. After allowing the culture to settle for 10 minutes, multiple antifungal disks were carefully placed on the surface of the agar to test susceptibility to different drugs.

9. The inoculated plates were incubated at room temperature for 24 hours to allow fungal growth and interaction with the antifungal agents.

10. After incubation, the plates were examined for clear zones of inhibition around the disks. The size and presence of these zones were used to assess the degree of resistance or sensitivity of *Candida* to each antifungal compound.

Extraction of Curcumin

1. Around five grams of fresh, peeled turmeric were carefully weighed using a clean scale. Gloves were worn throughout to maintain sterile conditions.

2. The turmeric was then chopped into smaller chunks to make the grinding process easier and more effective. A sterile blade was used for this step.

3. The pieces were transferred to a mortar, and about 50 millilitres of methanol were added. The mixture was ground thoroughly until a smooth, even paste was formed.

4. Once ground, the paste-like mixture was slowly poured into a sterile beaker. A spatula was used to scrape off any remaining bits from the mortar.

5. The entire mixture was passed through filter paper to remove any solid turmeric particles. This step took some time to ensure that the liquid extract was clear.

6. The filtered extract—containing the curcumin—was collected in a Falcon tube. It was then labelled properly and kept aside for use in later experiments.

Thin Layer Chromatography

Thin Layer Chromatography (TLC) is a simple yet effective technique used to separate and analyze components in a mixture. A small amount of the sample is applied to a plate coated with a thin layer of adsorbent material like silica gel or alumina. As the solvent moves up the plate by capillary action, it carries the sample components at different rates, depending on their polarity and interaction with the stationary phase.

TLC is widely used due to its speed, low cost, and minimal sample requirement. It helps monitor chemical reactions, identify substances, and assess purity. The results are typically made visible under UV light or by applying specific stains. Each substance has a unique Rf (retention factor) value, which aids in identification (Sharma et al. [1]).

RF = Reaction Factor

RF value = Band distance/ Total distance

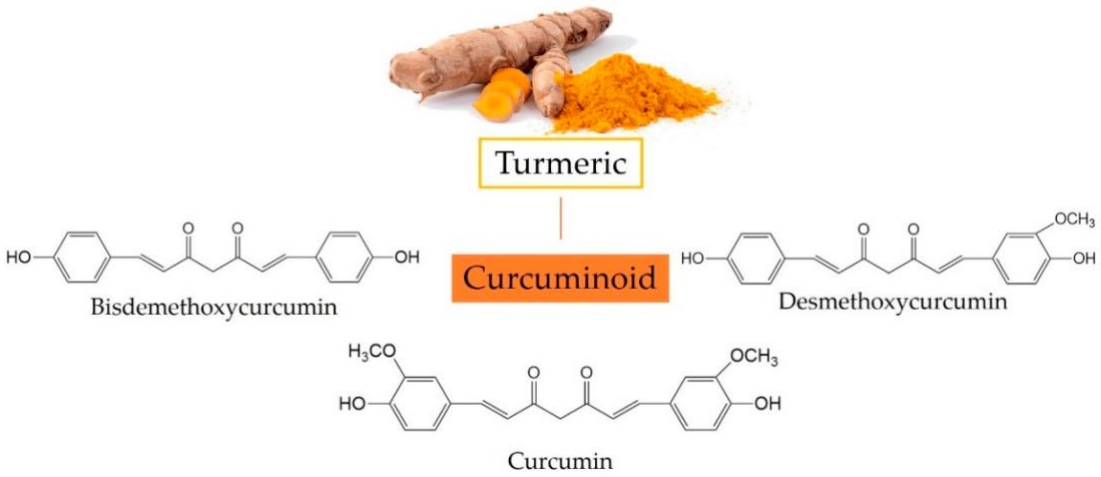

Fig: 1 – Structure of Curcumin: i) Bisdemothoxycurcumin; ii) Desmethoxycurcumin; iii) Curcumin.

Thin Layer Chromatography

1. A silica gel plate was carefully cut down to fit the size of the beaker being used. It was trimmed gently to avoid cracking or damaging the surface.

2. A straight baseline was drawn near the bottom edge of the plate using a pencil. This would serve as a guide for placing the sample spots.

3. Around 30 ml of solvent was mixed in a beaker using n-hexane, ethanol, and chloroform. The mixture was stirred lightly to combine properly.

4. Using a fine capillary tube, three tiny drops of the curcumin extract were gently spotted onto the baseline. Each drop was spaced out evenly to avoid overlapping.

5. The prepared silica plate was then placed upright inside the beaker, just enough for the solvent to touch below the sample line. The plate was left untouched.

6. Once the solvent had travelled far enough up the plate, it was taken out and left to dry. Under UV light, the movement of the curcumin bands became clearly visible.

7. The distance travelled by each band was measured, and the Rf values were calculated accordingly. This helped confirm the mobility and relative purity of the sample.

Synthesis of Nickel Nano particle

1. The process began by weighing out the required amount of nickel sulfate on a clean balance. This was done carefully to avoid any spillage or contamination.

2. About 50 ml of distilled water was poured into the beaker to dissolve the nickel sulfate. The solution was stirred briefly by hand to help it mix.

3. The beaker was then placed on a magnetic stirrer, and the solution was stirred continuously at 550 rpm while the temperature was held steady at around 45°C. This step helped the nickel dissolve properly.

4. In the meantime, 20 ml of previously extracted curcumin was gently boiled. Once heated, it was slowly added into the nickel solution drop by drop to avoid shocking the mixture.

5. To keep the reaction conditions just right, the pH was adjusted to stay between 8 and 9 using 5M NaOH. This was done gradually while checking the pH regularly.

6. The entire mixture was then stirred continuously for 3 hours to let the reaction complete. By the end, a thick, gel-like texture had started to form.

7. This gel was carefully scooped out and transferred into a clean Petri plate. Every tool used was sterile to avoid any contamination.

8. The Petri plate was left to sit undisturbed overnight at room temperature, allowing the mixture to dry out slowly on its own.

9. Once the mixture had fully dried, the solid layer was gently broken down using a clean mortar and pestle. Care was taken to crush it into a smooth, consistent powder without losing any material. This powdered form was then ready to be used for preparation.

10. The powdered nickel particles were then transferred into a fresh Eppendorf tube. The tube was labeled clearly to avoid mix-ups.

11. Around 1.5 ml of DMSO was added to the Eppendorf, followed by 1% of the powdered nickel mixture. The contents were mixed gently until fully blended.

12. The final solution was safely stored in the tube for later use in further experiments.

Siderophore synthesis

Siderophore synthesis is an essential survival mechanism used by microorganisms to obtain iron from environments where it is scarce or poorly available. Although iron is vital for processes like respiration and DNA synthesis, it often exists in insoluble ferric (Fe^{3+}) form under aerobic and neutral pH conditions. To access this iron, microbes release siderophores—small, high-affinity molecules that bind ferric ions and form stable complexes.

This process is tightly regulated and typically triggered when intracellular iron levels drop. Microorganisms produce structurally diverse siderophores, mainly classified as catecholates, hydroxamates, or mixed types based on their iron-binding groups. Beyond iron acquisition, siderophores also play roles in microbial competition, host colonization, and environmental adaptation. Their study has important implications in medical, agricultural, and industrial research, including developing antimicrobial agents, enhancing plant growth, and improving iron bioavailability in biotechnological processes.

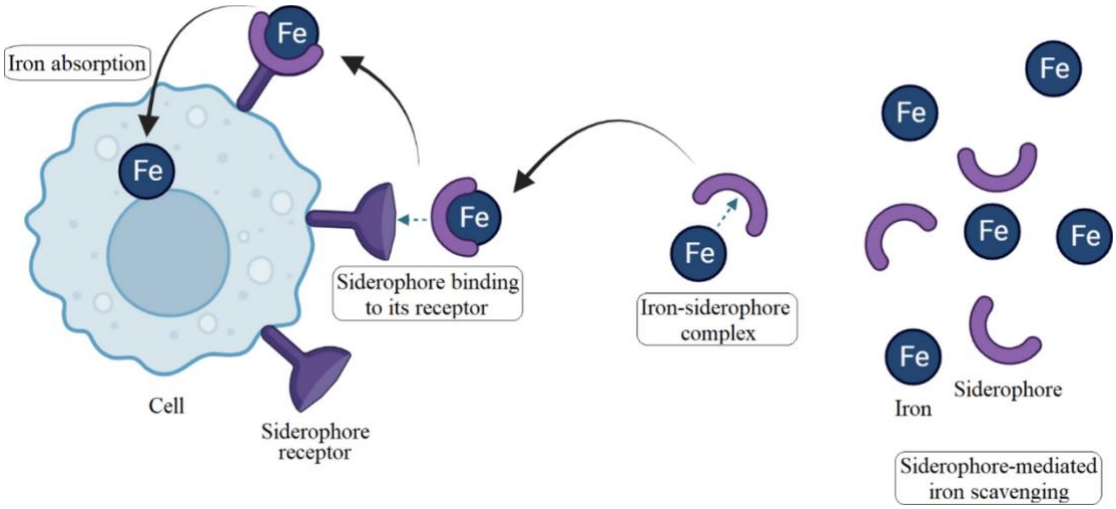

Fig: 2 – Iron chelating of Siderophore in Cell

Fish Gut Extraction Procedure

1. A fresh fish was picked up from the local market for this part of the study. It was chosen based on freshness and size, just enough to give a usable amount of gut material. Basic hygiene and handling precautions were followed to avoid contamination.

2. A simple saline solution was prepared by dissolving sodium chloride (NaCl) in clean, distilled water. The concentration was kept close to natural body conditions so it wouldn't damage the gut tissue. This solution was used later to gently rinse out the gut contents.

3. The fish was carefully cut open along the middle using a clean scalpel. The cut was made with steady hands to make sure none of the internal organs were unnecessarily damaged. Everything was done on a clean surface to keep things sterile.

4. The intestine was gently taken out using sterilized forceps. It was handled with care so it didn't tear or break during removal. Once removed, the gut was placed in a clean container for the next step.

5. Some of the prepared saline water was drawn into a sterile syringe. This was then slowly passed through the gut to flush out its contents. The liquid coming out was collected carefully into a clean Falcon tube.

6. The gut fluid that was flushed out was collected, making sure the sample wasn't lost or contaminated. Once collected, the tube was sealed and labelled properly.

7. The gut fluid was filtered using a membrane filter to remove any large particles or unwanted debris. This was done gently to avoid losing any important biological material. Finally, the clean filtrate was transferred into an Eppendorf tube and stored for future experiments.

Probiotic Siderophore Synthesis

1. A commercially available capsule containing *Lactobacillus rhamnosus* was selected for use in this experiment. The capsule was handled with clean, dry hands to prevent any form of contamination. It was brought to room temperature before opening to avoid moisture absorption (Kothari et al. [6]).

2. The capsule was carefully opened, and its contents were transferred into sterile liquid Nutrient Broth (NB). The mixture was gently swirled to ensure the bacteria were fully suspended in the broth. The culture was then incubated at room temperature for 24 hours to allow the probiotic strain to grow and multiply.

3. After incubation, the broth containing *L. rhamnosus* was passed through a sterile membrane filter to remove any clumps or larger particles. This step helped in obtaining a clean, uniform suspension of the probiotic. The filtered solution was then collected into a sterile Eppendorf tube and stored under appropriate conditions for use in later experiments.

Fig: 3 – *Lactobacillus rhamnosus* Capsules as Probiotic Siderophore Source

Bio polymer synthesis

Biopolymer synthesis is the process by which living organisms build long, chain-like molecules using smaller, repeating units found in nature. These materials—like proteins, DNA, or natural plastics—are made through enzyme-controlled reactions inside cells. Each small unit, whether it's a sugar, amino acid, or nucleotide, is carefully linked together in a specific sequence, depending on the function the final product needs to perform. This makes the process both precise and essential for life.

In natural settings, cells manage this process automatically, adjusting to internal signals or environmental changes like nutrient levels or stress. In applied science, researchers often mimic or enhance this natural system by using microbes or engineered cells to produce useful biopolymers on a larger scale. These materials have a wide range of uses, from eco-friendly packaging to medical implants, because they're biodegradable, biocompatible, and sustainable. The core idea behind their synthesis is simple: take small, natural building blocks and turn them into something much more complex and useful through controlled, step-by-step assembly.

Biopolymer Synthesis Procedure

1. A bacterial strain known for biopolymer production, such as *Bacillus subtilis* or *Pseudomonas aeruginosa*, was selected. The strain was either obtained from a lab culture or isolated from a natural source and confirmed using basic microbiological techniques.
2. A nutrient medium was prepared using carbon sources like glucose or molasses, along with essential salts and minerals. The composition was adjusted to favor biopolymer production over regular cell growth.
3. The selected bacterial culture was introduced into the sterile growth medium under aseptic conditions. The setup was placed in a shaker incubator to maintain proper temperature and oxygen supply for active fermentation.
4. The pH, temperature, and aeration were regularly monitored throughout the incubation period. In some cases, nitrogen or phosphate levels were deliberately limited to trigger biopolymer accumulation within the bacterial cells.
5. After sufficient growth and polymer buildup (usually after 48–72 hours), the bacterial culture was harvested. This was done by centrifuging the culture to separate the cells from the liquid medium.
6. The collected cell pellet was treated with hot water, detergent, or a mild chemical to break the cells open. This step helped release the intracellular biopolymer.
7. The lysed mixture was filtered or centrifuged again to remove debris. The supernatant containing the biopolymer was collected carefully for further processing.
8. A solvent such as ethanol, acetone, or chloroform was added to precipitate the biopolymer. The precipitate was then collected, washed to remove impurities, and air-dried or freeze-dried.
9. The dried biopolymer was weighed, labeled, and stored in a clean container for characterization and future applications.

Cup-plate assay of 5 Experimental factors against *Candida spp.*

1. About 250 mL of Sabouraud Dextrose Agar (SDA) was prepared in a clean conical flask. The medium was mixed thoroughly to ensure a uniform consistency. Standard lab safety and hygiene protocols were followed throughout the preparation.

2. The SDA and the required glassware were placed in the autoclave and sterilized at 15 PSI. The sterilization process was carried out for the recommended duration. This ensured that all microbial contaminants were eliminated.

3. Once autoclaving was completed, the sterilized glassware was carefully transferred into a laminar airflow cabinet. This was done under sterile conditions to avoid contamination. The workspace was kept sterile throughout.

4. The surface of the laminar flow hood was thoroughly wiped with disinfectant. This ensured a clean and contamination-free environment for the upcoming steps. All materials and instruments were arranged before use.

5. The sterile SDA was poured into autoclaved Petri plates and allowed to solidify at room temperature. The agar was poured evenly to create a smooth surface. Solidified plates were kept covered to avoid airborne contamination.

6. After solidification, 100 µL of a freshly prepared Candida suspension was pipetted into a sterile liquid culture tube. The suspension was gently mixed to ensure even distribution of fungal cells. This culture was then used for plating.

7. The Candida suspension was uniformly spread across the solidified SDA surface using a sterile glass spreader. A consistent layer was ensured for accurate testing. The plates were allowed to sit for a few minutes.

8. After 10 minutes, a well borer was sterilized using a spirit lamp and five wells were made in each agar plate. The wells were spaced evenly to prevent overlapping inhibition zones. Care was taken to maintain sterility during this step.

9. Into the five wells, 100 µL each of the test compounds was added. The compounds used were Curcumin, Nickel Nanoparticles, Fish Gut Extract, Probiotic Solution, and Biopolymer Extract. Each compound was pipetted into a separate, labeled well.

10. The inoculated plates were incubated at room temperature for 24 hours. During this time, the antifungal activity of each compound was allowed to take effect. The plates were covered and handled carefully to avoid contamination.

11. After the incubation period, zones of inhibition were observed around the wells. The diameters of these zones were measured to evaluate the effectiveness of each compound against Candida. All observations were recorded for analysis and comparison.

96 Well Plate Assay

The 96-well plate assay is a versatile and commonly used method in laboratories for analyzing multiple samples at the same time. Each well functions as an individual testing unit, which makes it easier to run many replicates or test different compounds side by side. Because only a small volume is needed per well—usually between 100 to 200 microliters—it's both cost-effective and efficient for large-scale experiments.

The wells are usually flat-bottomed to allow even light transmission during absorbance or fluorescence readings. Measurements are taken using a plate reader or spectrophotometer, depending on what kind of data is needed. Careful pipetting and consistent incubation conditions are key to getting reliable and reproducible results. The plate format allows for easy inclusion of controls, which helps verify the accuracy of experimental outcomes.

Fig: 4 – 96 Well Plate

Biofilm Eradication Assay

1. A fresh 96-well plate was taken and placed carefully inside the laminar air flow to maintain a sterile environment. This step helped reduce the chances of contamination. Everything, including tools and hands, was properly disinfected beforehand.

2. Using a micropipette, 200 µL of *Candida* cell culture was added to 12 individual wells. The pipetting was done slowly to avoid any bubbles. Each well was checked to make sure the culture settled properly.

3. Two of the wells were marked as control. These were left untreated to act as a baseline for comparison. The rest of the wells were used for testing the different compounds.

4. The entire plate was then incubated at room temperature for 48 hours. This allowed the *Candida* cells to settle and form biofilms on the well surfaces. The plate was not disturbed during this time.

5. After the incubation period, 20 µL of each test compound—curcumin, nickel nanoparticles, fish gut extract, probiotic, and biopolymer—was added to the other 10 wells (two wells for each factor). The compounds were pipetted gently to avoid breaking the biofilm. The two control wells were left untouched.

6. The plate was again incubated at room temperature for 24 hours to let the compounds interact with the biofilms. This step was important to check how each compound affected the formed biofilms. No movement or shaking was done during this time.

7. After 24 hours, the plate was flipped upside down over a waste container to discard the contents of the wells. This was done slowly to avoid dislodging any remaining biofilm. The plate was then gently tapped on a tissue to remove excess liquid.

8. Next, 200 µL of 1% Gram's crystal violet stain was added to all 12 wells. The stain was used to highlight the presence of biofilm by binding to it. The plate was left at room temperature for 15 minutes.

9. Once the staining time was over, the plate was again flipped to discard the excess stain. The wells were gently tapped on tissue to clear any remaining liquid. This helped reduce background noise during the reading.

10. Then, 200 µL of 30% acetic acid was added to each well to wash out the bound crystal violet. The acid helped release the dye from the biofilm so it could be measured later. The wells were left to sit for a short time to ensure complete destaining.

11. Each of the five test compounds was also added to separate blank wells containing only Sabouraud Dextrose Broth (SDB) and sterile water. These blanks were needed to account for the color or absorbance of the compounds themselves. This step made the final results more accurate.

12. Finally, the absorbance from each well was measured using an ELISA reader. The data showed how much biofilm was left after treatment. By comparing the treated wells with the controls, the effectiveness of each compound could be clearly observed.

Anti-biofilm Assay

1. A clean, sterile 96-well plate was brought inside the laminar air flow to maintain aseptic conditions. The workspace was properly disinfected beforehand, and all tools were handled carefully. This helped ensure that no contamination interfered with the experiment.

2. Using a micropipette, 200 µL of *Candida* cell culture was gently added to 12 individual wells. Each well was then briefly checked to make sure the culture was evenly spread.

3. Out of the 12 wells, two were marked as controls and left untreated. These served as a reference to compare how the test compounds affected biofilm formation. The remaining 10 wells were used for treatment with different factors.

4. Into the other 10 wells, 20 µL of each of the five selected compounds Curcumin, Nickel nanoparticles, Fish gut extract, Probiotic, and Biopolymer was. Each compound was added to two wells to maintain consistency. Care was taken not to disturb the initial layer of *Candida* cells.

5. The plate was then left to incubate at room temperature for 48 hours. During this time, biofilms were expected to form within the wells. The plate was kept undisturbed throughout the incubation period.

6. After 48 hours, the plate was gently turned upside down to discard the liquid contents. This step was done slowly to prevent any disruption of the biofilm attached to the well surfaces.

7. Following that, 200 µL of 1% Gram's crystal violet stain was carefully added to each of the 12 wells. The stain was allowed to sit and bind to any remaining biofilm structures. It helped make the biofilm visible for further observation.

8. The plate was then kept at room temperature for about 15 minutes to allow proper staining. This step ensured that even thin layers of biofilm would be highlighted.

9. Once staining was complete, the plate was flipped again to discard the excess crystal violet.

10. After that, 200 µL of 30% acetic acid was added to all wells to release the bound crystal violet into solution. This made it possible to quantify the amount of biofilm indirectly. The solution was mixed gently by hand for a few seconds.

11. In separate wells, each of the test compounds was also added into blanks containing only Sabouraud Dextrose Broth (SDB) and sterile water. These blanks helped identify any color interference caused by the compounds themselves. It ensured that the absorbance readings would reflect only the presence of biofilm.

12. Finally, the results of the anti-biofilm assay were recorded using an ELISA reader. The absorbance values helped show how much biofilm had formed—or been reduced—after treatment with each compound. By comparing these values with the control wells, the effect of each factor could be clearly observed.

MTT Assay

1. A new sterile 96-well plate was carefully placed inside the laminar air flow to begin the assay under contamination-free conditions.

2. Using a micropipette, 100 microlitres of Candida cell culture were gently added into 12 separate wells. The pipetting was done slowly to avoid creating bubbles or disturbing the cell suspension. After filling, each well was checked to confirm even distribution of the culture.

3. Out of the 12 wells, two were marked as untreated controls. These control wells were important for comparing the effect of the treatments later on. No additional substances were added to these wells during the initial setup.

4. For the remaining 10 wells, 10 microlitres of the five different test factors—Curcumin, Nickel nanoparticles, Fish gut extract, Probiotic, and Biopolymer—were added in duplicate.

5. Once all the wells were set up, the plate was incubated at room temperature for 24 hours. During this time, the cells were allowed to grow and interact with the added substances. The plate was kept undisturbed to maintain consistent conditions across all wells.

6. After 24 hours, 100 microlitres of MTT reagent were added into each of the 12 wells. The reagent was pipetted gently to avoid disrupting the cells. This step was crucial for checking cell viability, as living cells would reduce the MTT into a purple-colored formazan compound.

7. The plate was then incubated again at room temperature for 4 hours to allow the reaction to take place. During this period, any viable Candida cells would convert the yellow MTT reagent into purple crystals. The more intense the purple color, the more metabolically active cells remained.

8. Once the incubation was over, the plate was carefully turned upside down to discard the liquid contents. This was done slowly so that the formazan crystals formed at the bottom of each well remained undisturbed.

9. Then, 100 microlitres of DMSO were added to each well to dissolve the purple formazan crystals. This allowed the dye to become measurable in a liquid form. Each well gradually turned purple, indicating the presence of viable cells.

10. The plate was incubated again at room temperature for 15 minutes to ensure full solubilization of the dye. During this time, the wells were not moved to prevent uneven mixing. By the end of this step, the colour was consistent and ready for measurement.

11. The absorbance values of all wells were then recorded using an ELISA reader. These readings showed how much cell activity remained after treatment with each compound. Comparing the treated wells with the control ones made it easier to understand how effective each substance was.

12. Lastly, any remaining DMSO was discarded by gently flipping the plate upside down again. The wells were carefully tapped out and the plate was set aside for disposal. All observations and results were documented for analysis.

MIC Assay

Factors dilution

- Curcumin:
    - Take 5 sterile microcentrifuge tube (MCT)
    - Mark on the lid

      ½, ¼, ⅛, 1/16, 1/32

    - Pipette out 10 micro-litre curcumin factor from crude 30ppendorf into ½ marked MCT and add 10 micro-litre of distilled H2O
    - Invert mix the MCT.
    - Similarly pipette out 10 micro-litre of dilution from ½ marked MCT into ¼ marked MCT and add 10 micro-litre of distilled H2O. Invert mix the MCT
    - Repeat the process till 1/32 marked MCT

- Nickel nano particle
    - Take 5 sterile microcentrifuge tube (MCT)
    - Mark on the lid

      ½, ¼, ⅛, 1/16, 1/32

    - Pipette out 10 micro-litre of nickel nano particle factor from crude 30ppendorf into ½ marked MCT and add 10 micro-litre of distilled H2O
    - Invert mix the MCT.
    - Similarly pipette out 10 micro-litre of dilution from ½ marked MCT into ¼ marked MCT and add 10 micro-litre of distilled H2O. Invert mix the MCT
    - Repeat the process till 1/32 marked MCT

- Fish gut
    - Take 5 sterile microcentrifuge tube (MCT)
    - Mark on the lid

      ½, ¼, ⅛, 1/16, 1/32

- Pipette out 10 micro-litre fish gut factor from crude 31ppendorf into ½ marked MCT and add 10 micro-litre of distilled H2O
- Invert mix the MCT.
- Similarly pipette out 10 micro-litre of dilution from ½ marked MCT into ¼ marked MCT and add 10 micro-litre of distilled H2O. Invert mix the MCT
- Repeat the process till 1/32 marked MCT

- Probiotic
  - Take 5 sterile microcentrifuge tube (MCT)
  - Mark on the lid

    ½, ¼, ⅛, 1/16, 1/32
  - Pipette out 10 micro-litre of probiotic factor from crude 31ppendorf into ½ marked MCT and add 10 micro-litre of distilled H2O
  - Invert mix the MCT.
  - Similarly pipette out 10 micro-litre of dilution from ½ marked MCT into ¼ marked MCT and add 10 micro-litre of distilled H2O. Invert mix the MCT
  - Repeat the process till 1/32 marked MCT

- Biopolymer

- Take 5 sterile microcentrifuge tube (MCT)
- Mark on the lid

  ½, ¼, ⅛, 1/16, 1/32
- Pipette out 10 micro-litre biopolymer factor from crude 31ppendorf into ½ marked MCT and add 10 micro-litre of distilled H2O
- Invert mix the MCT.
- Similarly pipette out 10 micro-litre of dilution from ½ marked MCT into ¼ marked MCT and add 10 micro-litre of distilled H2O. Invert mix the MCT
- Repeat the process till 1/32 marked MCT

1. A new 96-well plate was placed carefully inside the laminar air flow to maintain sterile conditions. All steps were carried out under aseptic precautions.

2. Using a micropipette, 100 microlitres of *Candida* cell culture were added to 30 wells. The culture was distributed slowly to avoid bubble formation.

3. In the first row of five wells, 10 microlitres of each crude factor were added—Curcumin, Nickel nanoparticles, Fish gut extract, Probiotic, and Biopolymer. Each factor was pipetted into its designated well.

4. In the second row, 10 microlitres of a ½ dilution of each crude factor were added to their respective wells. Fresh dilution was prepared for each.

5. The third row received 10 microlitres of the next ½ dilution from the previous set. The factors were added carefully to maintain the gradient.

6. Similarly, the fourth row was filled with the next level of ½ dilution. All additions were done with separate tips to avoid cross-contamination.

7. In the fifth row, the dilution process was repeated, and 10 microlitres were added as before. The same order of factors was followed.

8. The sixth and final row was treated with another round of ½ dilution, with 10 microlitres added into each well. Dilutions were mixed properly before pipetting.

9. The plate was then incubated at room temperature for 24 hours. It was kept undisturbed during this time.

10. After incubation, the plate was taken out and read in the ELISA reader. Results were observed to determine the minimum inhibitory concentration.

Genomic DNA Extraction

1. 1.5 mL of *Candida* culture was transferred into a sterile microcentrifuge tube. The tube was spun at 10,000 rpm for 2 minutes to separate the cells, resulting in a pellet at the bottom. The supernatant was carefully removed, and 600 µL of TE buffer (composed of 10 mM Tris-HCl and 1 mM EDTA, pH 8.0) was added to gently resuspend the pellet.

2. To begin breaking down the fungal cell walls, 30 µL of lyticase enzyme (1 mg/mL concentration) was added to the tube. The contents were mixed gently to ensure even distribution. This mixture was then allowed to sit at room temperature for 10 minutes to promote enzymatic digestion.

3. Following this, 30 µL of proteinase K (1 mg/mL) was introduced to help degrade any proteins present. To further aid in breaking the cell membranes, 50 µL of 10% SDS was added. The tube was then incubated at 55°C for 20 minutes to ensure complete cell lysis and protein breakdown.

4. For protein precipitation, 100 µL of 3 M potassium acetate was added to the solution and mixed thoroughly. This helped in clumping unwanted proteins and cell debris together. The mixture was then prepared for further purification.

5. The sample was placed on ice for 10 minutes to enhance protein precipitation and stabilize the solution before centrifugation.

6. Centrifugation was carried out at 10,000 rpm for 10 minutes at 4°C to effectively separate the pellet from the clear liquid (supernatant).

7. The upper clear supernatant, which contained the DNA, was carefully transferred to a fresh microcentrifuge tube without disturbing the pellet.

8. To the new tube, an equal volume of a Phenol:Chloroform:Isoamyl alcohol mixture (25:24:1) was added for phase separation. This helped in removing proteins and other organic contaminants.

9. The mixture was gently inverted for 2 minutes to allow thorough mixing, followed by centrifugation at 10,000 rpm for another 10 minutes.

10. The upper aqueous phase was carefully collected into a new tube. An equal volume of Chloroform:Isoamyl alcohol (24:1) was then added to further clean the DNA.

11. This solution was again gently inverted for 2 minutes and spun down at 10,000 rpm for 10 minutes to complete the purification step.

12. The top aqueous layer was once more transferred carefully. To this, an equal volume of chilled isopropanol was added to precipitate the DNA.

13. The tube was then kept at -20°C for 30 minutes to allow complete precipitation of DNA.

14. After chilling, the sample was centrifuged again at 10,000 rpm for 10 minutes to pellet the DNA.

15. The isopropanol was discarded carefully without disturbing the DNA pellet.

16. To wash the pellet, 70% ethanol was added. This step helped remove any remaining salts or impurities.

17. Another round of centrifugation at 10,000 rpm for 10 minutes was performed to settle the DNA.

18. The ethanol was removed, and the pellet was left to air dry completely, ensuring that no residual alcohol remained.

19. Finally, the dried DNA pellet was dissolved in 20 µL of 1X TE buffer to rehydrate the DNA.

20. To remove any residual RNA contamination, 5 µL of Rnase was added to the tube, and it was incubated at 42°C for 45 minutes.

DNA Damage Study of *Candida*

1. Culturing and Treatment of Candida Cells

   Candida strains were initially grown in SBD (Sabouraud Dextrose Broth) medium at 30°C until reaching the mid-logarithmic phase of growth. Once this stage was achieved, the culture was divided into multiple portions. Each aliquot was treated with one of the following: Curcumin extract, Nickel nanoparticles, siderophores derived from fish gut microbiota, probiotic-derived siderophores, or a specific biopolymer. These treatments were administered independently to assess their individual effects. All treated samples were incubated for an additional 2 hours under the same temperature conditions. A control sample, left untreated, was maintained to serve as a baseline for comparison.

2. Harvesting Cells and Extracting DNA

   Following treatment, cells were collected by centrifuging the cultures at 5,000 × g for 5 minutes. The resulting cell pellets were rinsed twice with sterile phosphate-buffered saline (PBS) to remove residual media and unbound substances, ensuring a clean sample for DNA extraction.

3. Preparation of PCR Reactions

   Each sample underwent polymerase chain reaction (PCR) in a final reaction volume of 25 µL. The reaction mix for each tube included:
   PCR mixture 25 micro-litre
   1. Master mix 12.5microliter (Taq polymerase+dNTPs+ Mg2+)
   2. H2O - 10microlitre
   3. Template – 1 micro-litre
   4. Primer – 1.5 micro-litre
   The same primer pair, specific to the target gene under investigation, was used consistently across all samples to ensure uniformity in amplification.

4. PCR Thermal Cycling Conditions

Amplification was carried out using a thermal cycler under the following conditions:

• Initial denaturation at 95°C for 5 minutes

• 35 cycles of:

o Denaturation at 95°C for 30 seconds

o Primer annealing at 55°C for 30 seconds

o DNA extension at 72°C for 1 minute

• Final elongation at 72°C for 10 minutes

These parameters were selected to optimize the specificity and yield of the target DNA fragments.

5. Agarose Gel Electrophoresis

- The amplified PCR products were separated using 1.5% agarose gels pre-stained with ethidium bromide.

- Electrophoresis was performed at 100 volts for approximately 45 minutes using 1X TAE buffer.

- DNA bands were visualized under ultraviolet (UV) illumination and recorded using a gel documentation system.

Stress Profiling of Protein Expression

1. Preparation of Reagents and Buffers

a. Sample Preparation Buffer (Laemmli Buffer)

- 62.5 mM Tris-HCl (pH 6.8)
- 2% SDS (sodium dodecyl sulfate)
- 10% Glycerol
- 0.01% Bromophenol Blue (tracking dye)
- 5% β-mercaptoethanol or 100 mM DTT (as reducing agents)

  Used to denature proteins and ensure uniform negative charge distribution across samples.

b. Resolving Gel Buffer

- 1.5 M Tris-HCl (pH 8.8)
- 0.4% SDS

  Forms the lower gel layer responsible for molecular weight-based separation.

c. Stacking Gel Buffer

- 0.5 M Tris-HCl (pH 6.8)
- 0.4% SDS

  Concentrates proteins before they enter the resolving gel for precise banding.

d. Running Buffer (1X Tris-Glycine-SDS Buffer)

- 25 mM Tris
- 192 mM Glycine
- 0.1% SDS
- pH ~8.3

  Enables efficient current conduction during electrophoresis.

e. Polyacrylamide Solution

- Acrylamide and Bis-acrylamide in a 30:0.8 ratio
- 10% gel for 20–100 kDa proteins
- 12–15% gel for smaller proteins

  Gel concentration is selected based on the molecular weight range of target proteins.

f. Gel Polymerization Agents

- 10% Ammonium Persulfate (APS)
- TEMED (Tetramethylethylenediamine)

  These agents catalyze polymerization of the acrylamide matrix.

2. Gel Casting

a. Resolving Gel Preparation

   Components for the resolving gel are mixed and poured into a gel cassette until it fills about two-thirds of the space. A layer of isopropanol or distilled water is gently overlaid to prevent oxygen interference. Polymerization is allowed for 20–30 minutes.

b. Stacking Gel Preparation

   Once the resolving gel has solidified, the overlay is removed. The stacking gel solution is poured, and a clean comb is inserted to create wells. This layer is left to polymerize for another 15–20 minutes.

4. Sample Preparation

Protein samples are mixed with Laemmli buffer in a 1:1 ratio and incubated at 95°C for 5 minutes to denature the proteins. Following this, a brief centrifugation step ensures all contents are collected at the bottom of the tube.

5. Electrophoresis

    a. Sample Loading

    The polymerized gel is mounted in the electrophoresis chamber and submerged in 1X running buffer. Prepared samples, along with a molecular weight marker, are carefully loaded into the wells.

    b. Running Conditions

    Electrophoresis begins at 80V for the stacking gel phase and is increased to 100–120V for the resolving gel. The run continues for approximately 1.5 to 2 hours or until the dye front nears the gel's base.

6. Staining and Visualization

    a. Coomassie Staining

    The gel is submerged in a 0.1% Coomassie Brilliant Blue solution (R-250 or G-250) prepared in methanol and acetic acid. It is gently agitated for one hour to allow even staining.

    b. Destaining

    Excess stain is removed using a destaining solution containing methanol, acetic acid, and water. This process typically takes 4–6 hours or may be left overnight for optimal clarity.

7. Documentation and Analysis

    - The gel is observed under white light, and band patterns are captured using a gel documentation system.
    - Protein bands are compared against the molecular weight marker for size estimation and expression analysis.

Result and Discussion

- ## Isolation of *Candida* Species

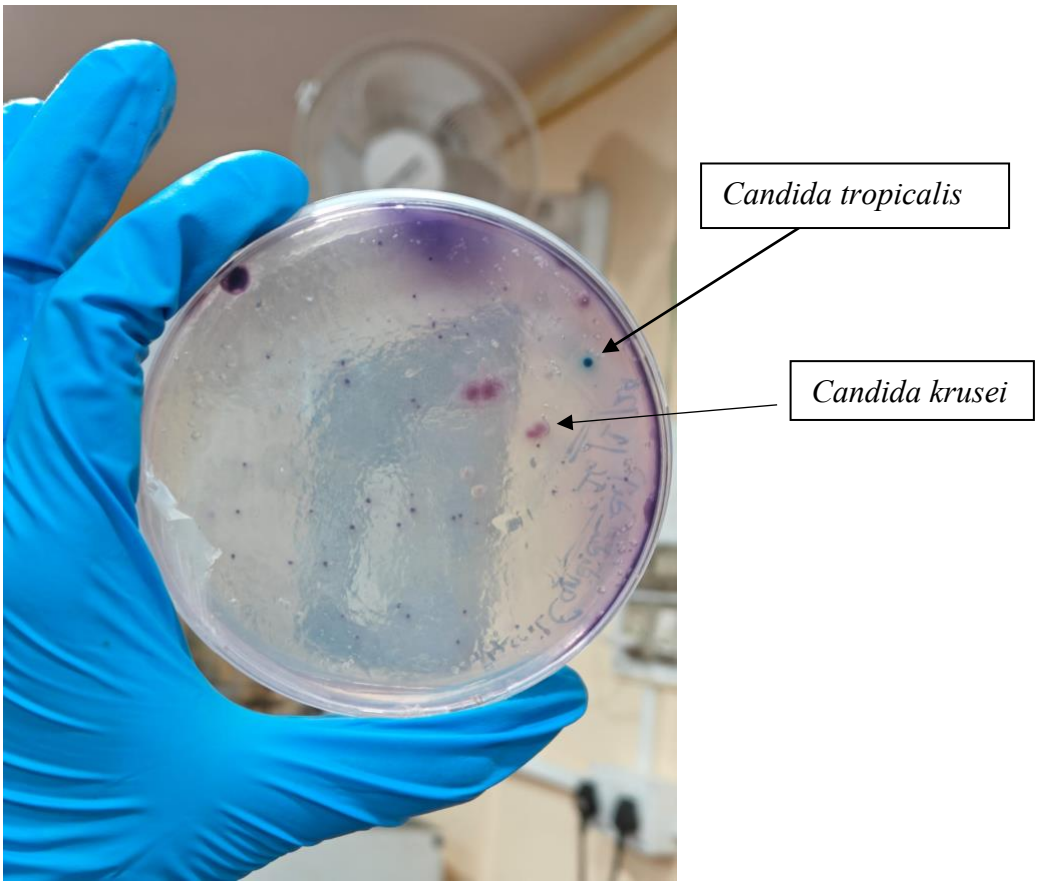

Fig: 5 – Isolation of *Candida spp.* On *Candida* Differentiate Agar Plate

- **Multi Drug Resistant Assay**

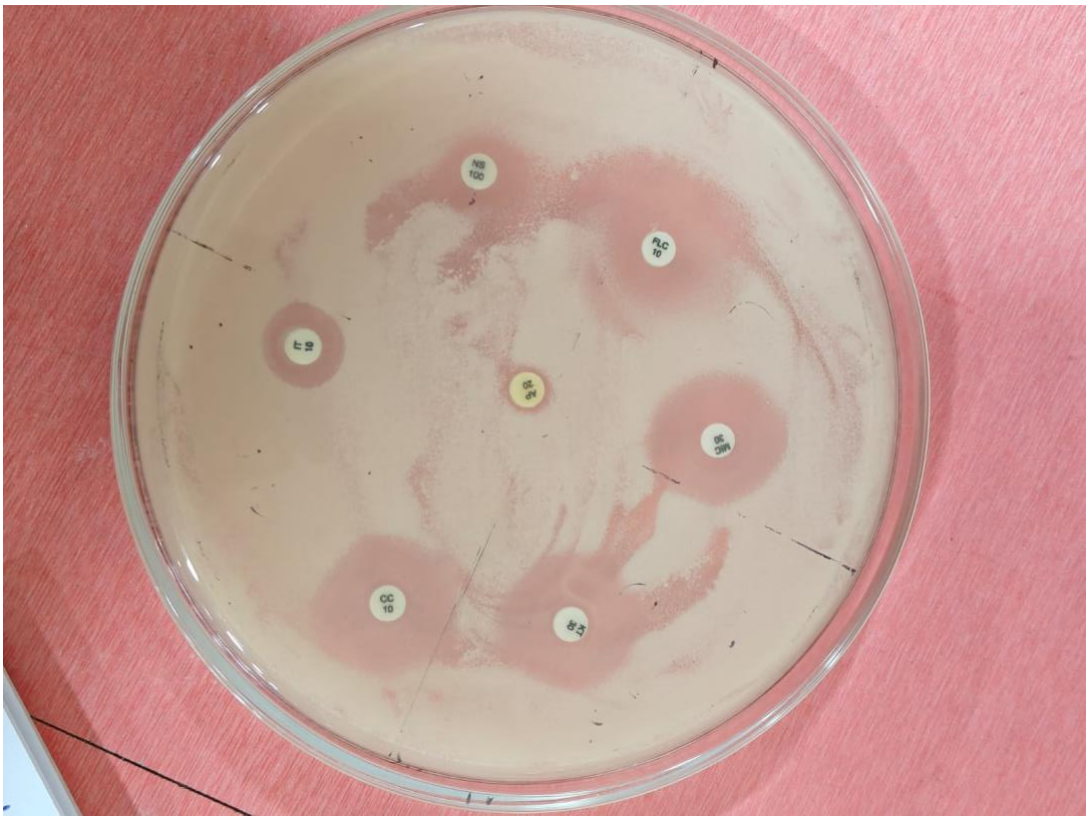

Fig: 6 – Multi Drug Resistant Assay against *Candida spp.*

Antifungal Drug	Zone of Inhibition (ZOI)	Antifungal Drug	Zone of Inhibition (ZOI)
Amphotericin B (AP)	1 cm	Fluconazole (FLC)	3 cm
Nystatin (NS)	1.7 cm	Miconazole (MIC)	2.5 cm
Itraconazole (IT)	1.5 cm	Ketoconazole (KT)	2.5 cm
Clotrimazole (CC)	2.5 cm		

Table: 1 – Antifungal Drugs and their ZOI against *Candida spp.*

- **Extraction of Methanolic Curcumin**

Fig: 7.1 – Filtration of Methanolic Curcumin

Fig: 7.2 – Boiling of Methanolic Curcumin

- **Thin Layer Chromatography**

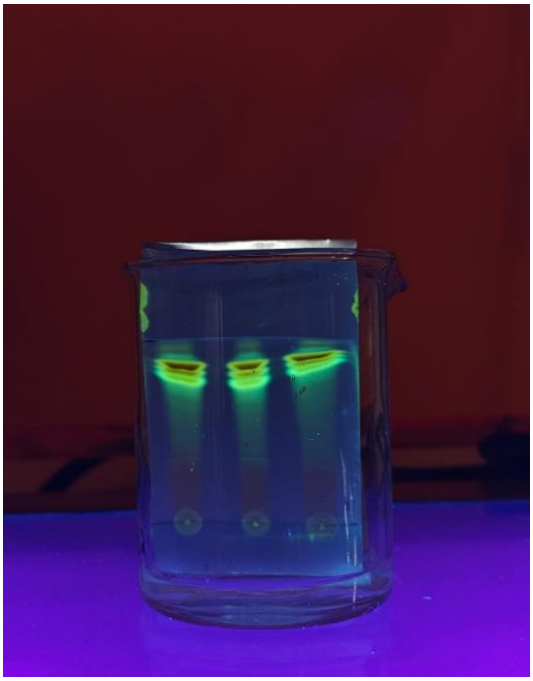

Fig: 7.3 – Thin Layer Chromatography under UV Light

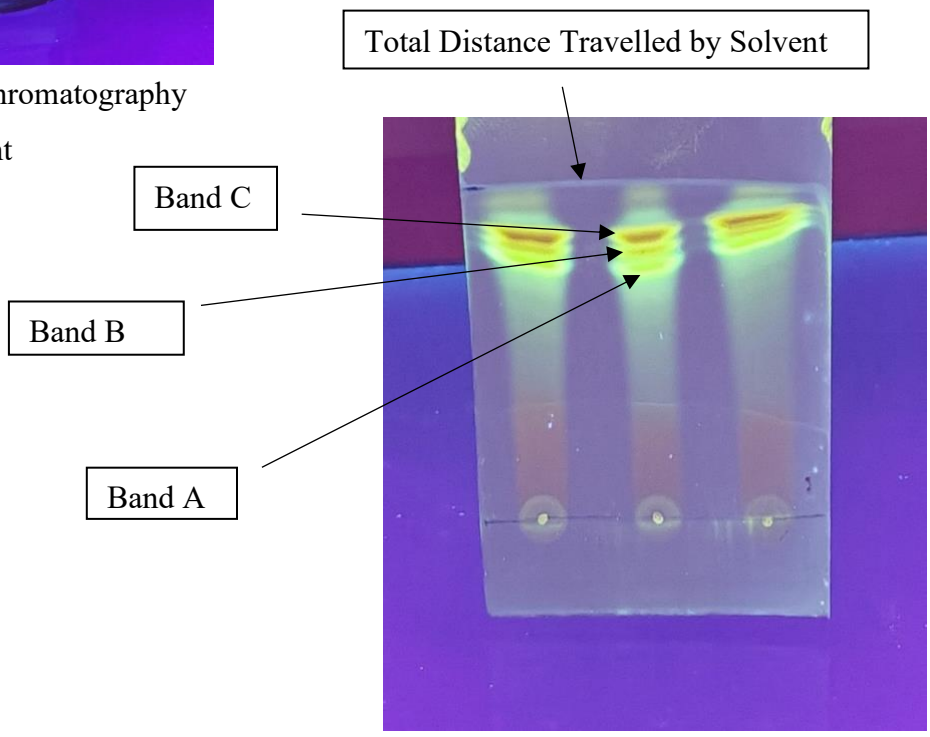

Fig: 7.4 – Band Distance travelled through Solvent

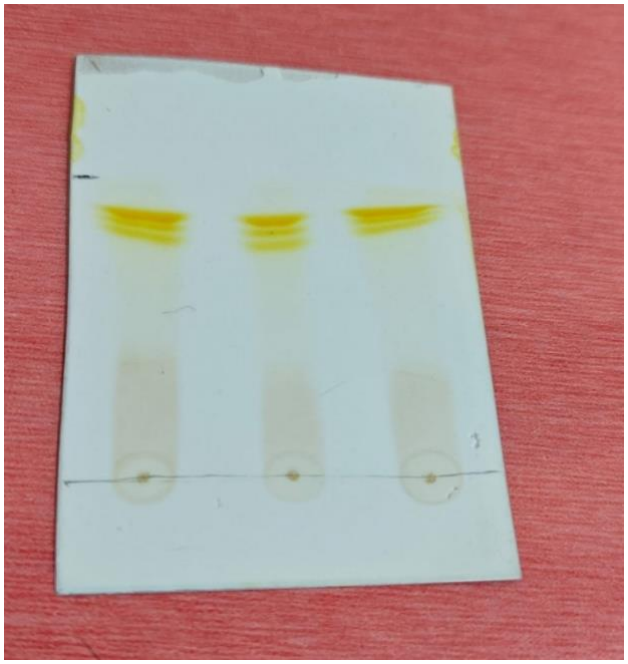

Fig: 7.5 – Thin Layer Chromatography by Capillary Action

Band	Band Distance	RF Value	
Band A	2.8 cm	$\frac{2.8}{3.8}$	0.736
Band B	3 cm	$\frac{3}{3.8}$	0.789
Band C	3.2 cm	$\frac{3.2}{3.8}$	0.842

Table: 2 – Calculation of RF Value from Thin Layer Chromatography

- **Synthesis of Nickel Nano Particle**

Fig: 8.1 – Nickel Nano Particle Synthesis

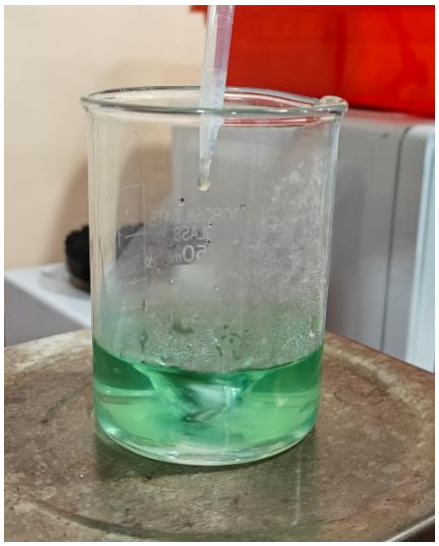

Fig: 8.2 – Nickel Nanoparticle after drop wise addition of Curcumin Boil

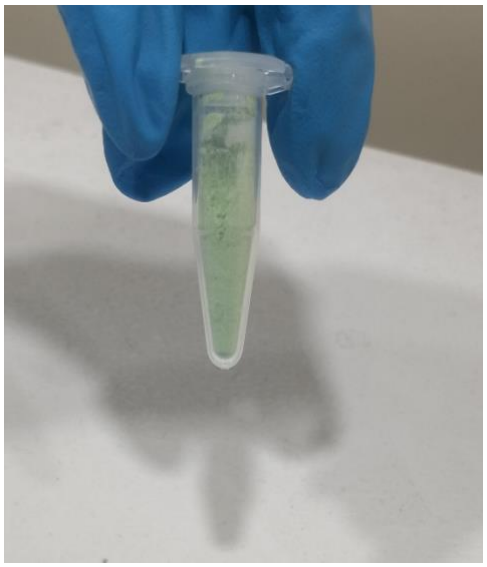

Fig: 8.3 – Grounded form of dried Nickel Nano Particle

Fig: 8.4 – Grounded form of Synthesized Nickel Nano Particle

- **UV Spectra Visualization of Synthesized Nickel Nanoparticle**

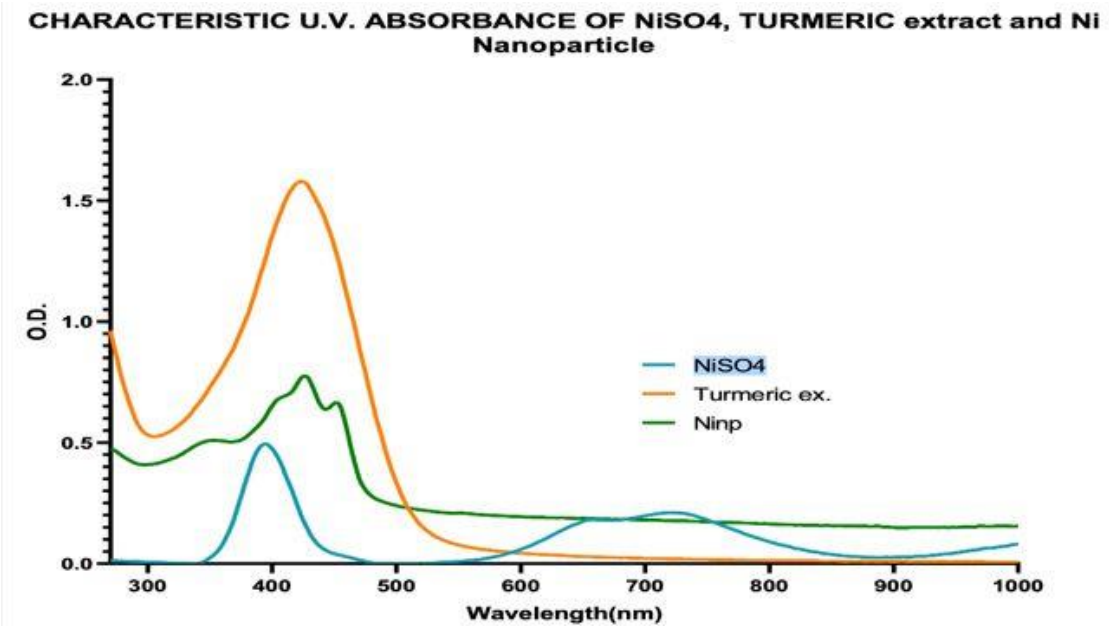

Fig: 8.5 - UV-Vis Absorbance of $NiSO_4$, Turmeric Extract, and Ni Nanoparticle

The UV-Vis absorbance spectrum displays a pronounced peak shift and intensity variation among $NiSO_4$, turmeric extract, and the synthesized Ni nanoparticles. The broadened peak for NiNPs, particularly between 400–500 nm, confirms successful nanoparticle formation with surface modification by curcumin. This optical signature also suggests increased stability and potential bioactivity relevant to antifungal applications.

- **Siderophore Synthesis**

Fish Gut Siderophore

Fig: 9.1 – Cutting of Fish Interstine

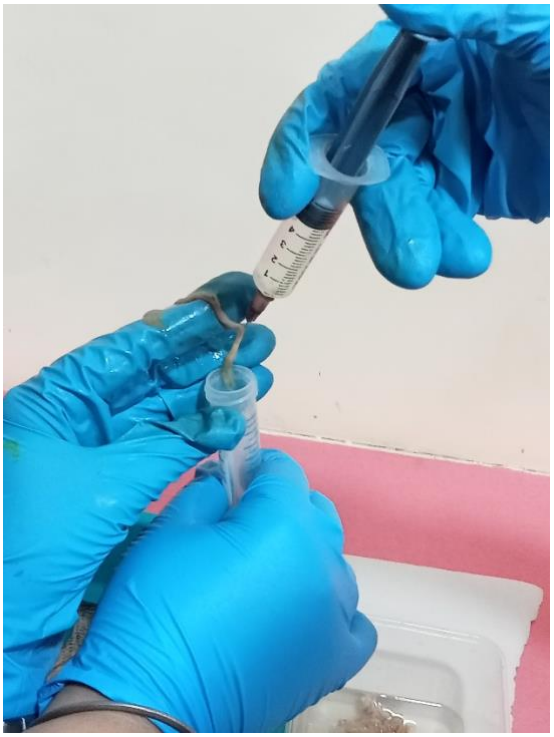

Fig: 9.2 – Fish Gut Siderophore collection

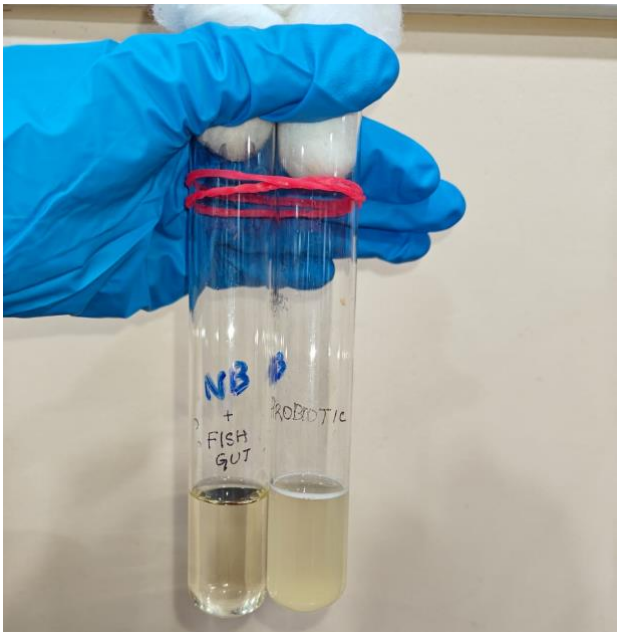

Fig: 9.3 – Fish Gut Siderophore and Probiotic Siderophore Synthesis

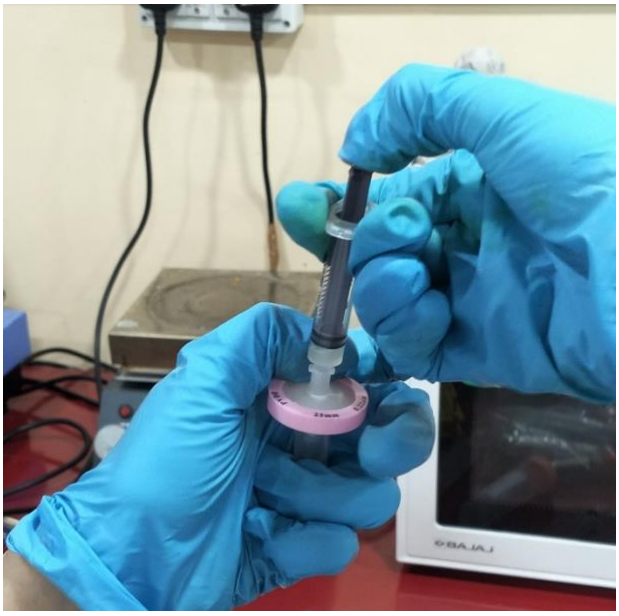

Fig: 9.4 – Membrane Filtration of Fish Gut

Fig: 9.5 - Membrane Filtration of Probiotic Siderophore

- **Biopolymer**

Synthesis

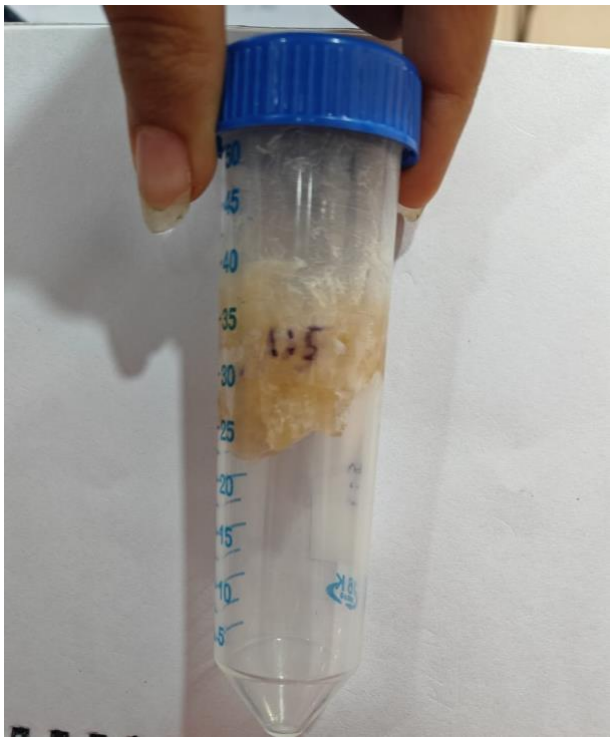

Fig: 10 – Synthesized Biopolymer – Hyperbranched polymer of polyethylene glycol and Citric acid mixed in 1:5 ratio.

- **Cup Plate Assay of 5 Experimental Factors against *Candida spp*.**

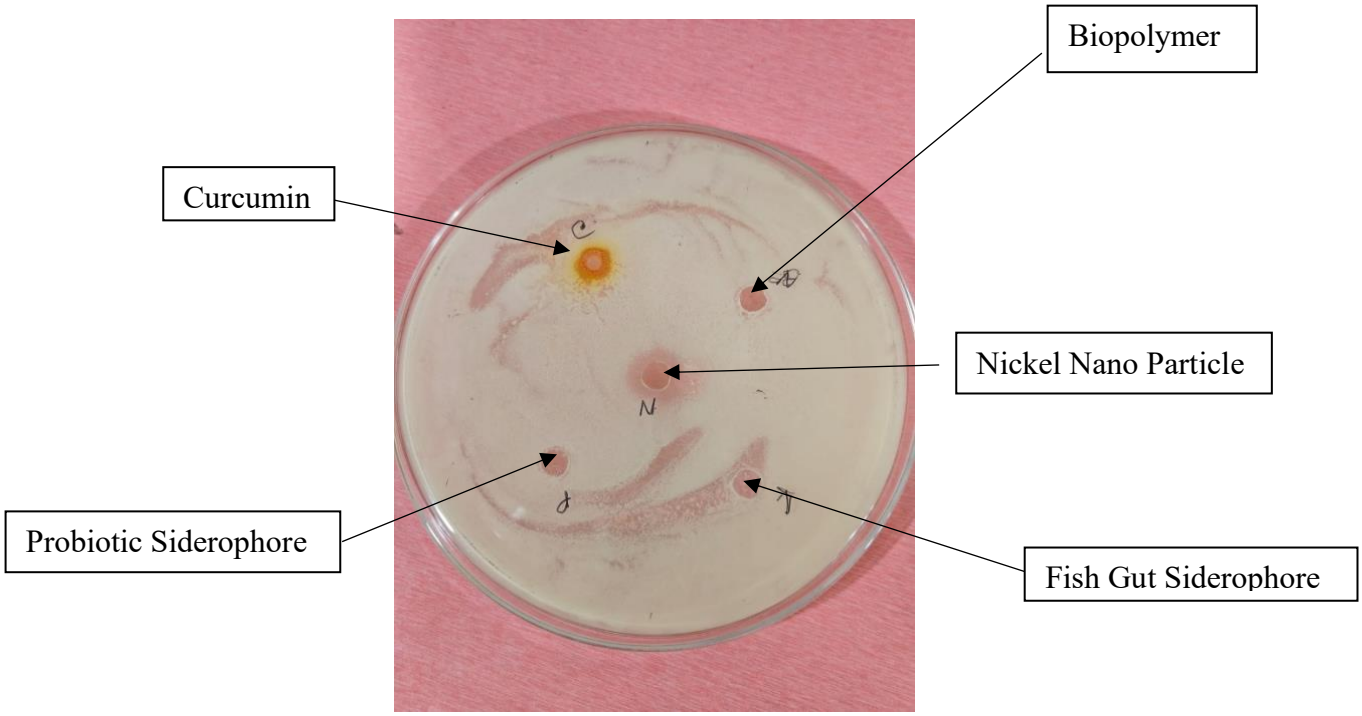

Fig: 11 - Cup Plate Assay of 5 Experimental Factors against Candida spp.

Experimental Factors	Zone Of Inhibition (ZOI)
Curcumin (C)	1.2 cm
Nickel Nano Particle (N)	2 cm
Fish Gut Siderophore (F)	1.2 cm
Probiotic Siderophore (P)	No zone
Biopolymer (B)	No zone

Table: 3 – 5 Experimental Factors and their ZOI against *Candida spp.*

- **96 Well Plate Assay**

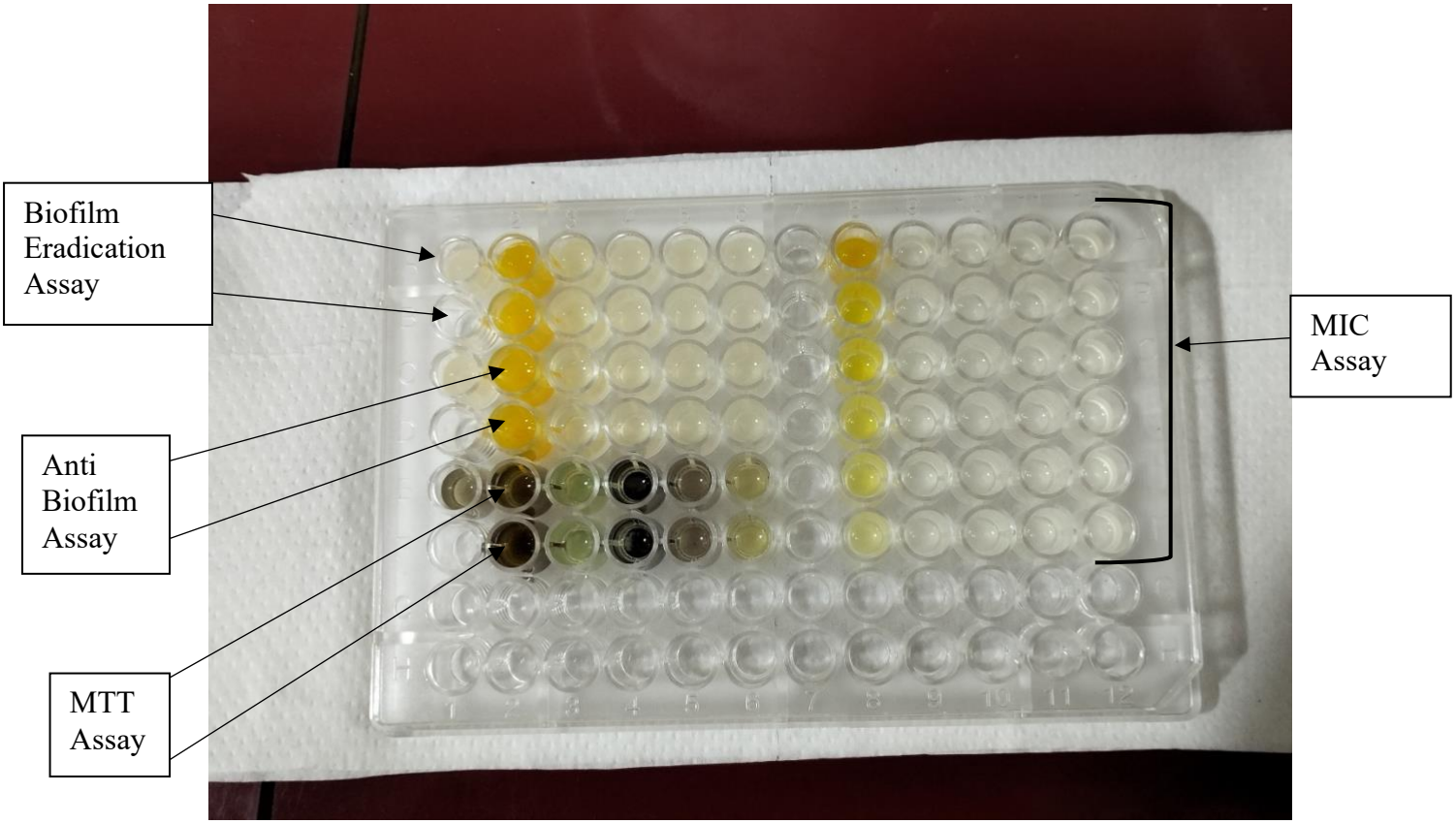

Fig: 12.1 – *Candida* cells and Experimental Factors in 96 Well Plate

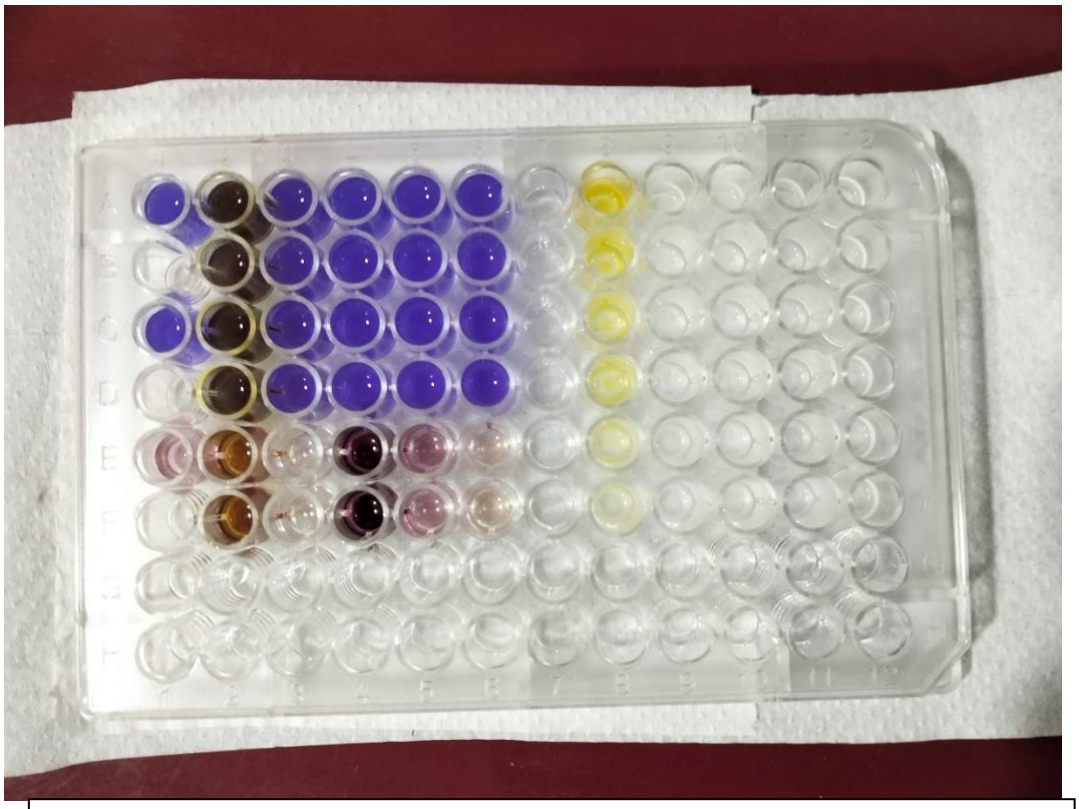

Fig: 12.2 – 96 Well Plate after staining with Crystal violet and DMSO

- **Biofilm Eradication Assay**

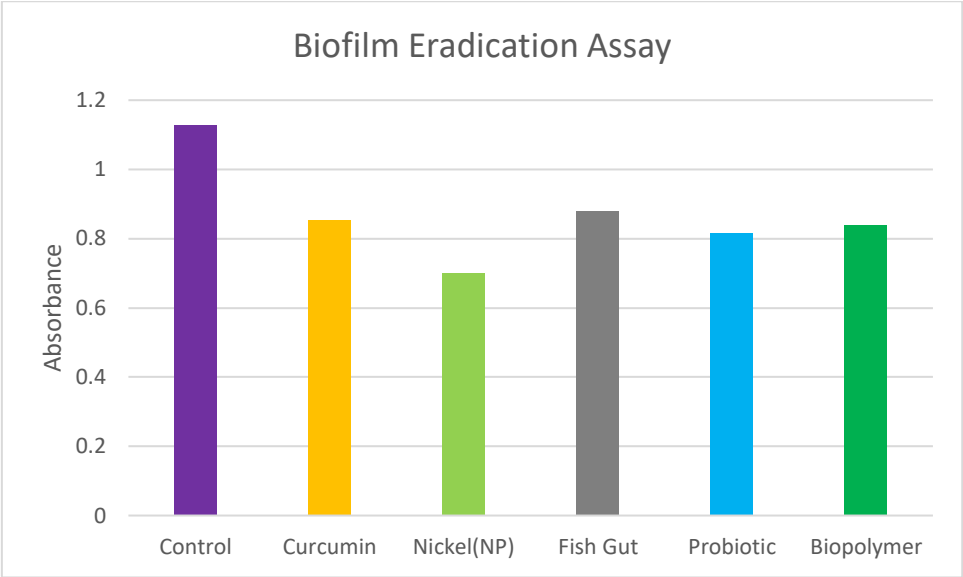

Fig: 12.3 – Absorbance graph of Biofilm Eradication Assay

- **Antibiofilm Assay**

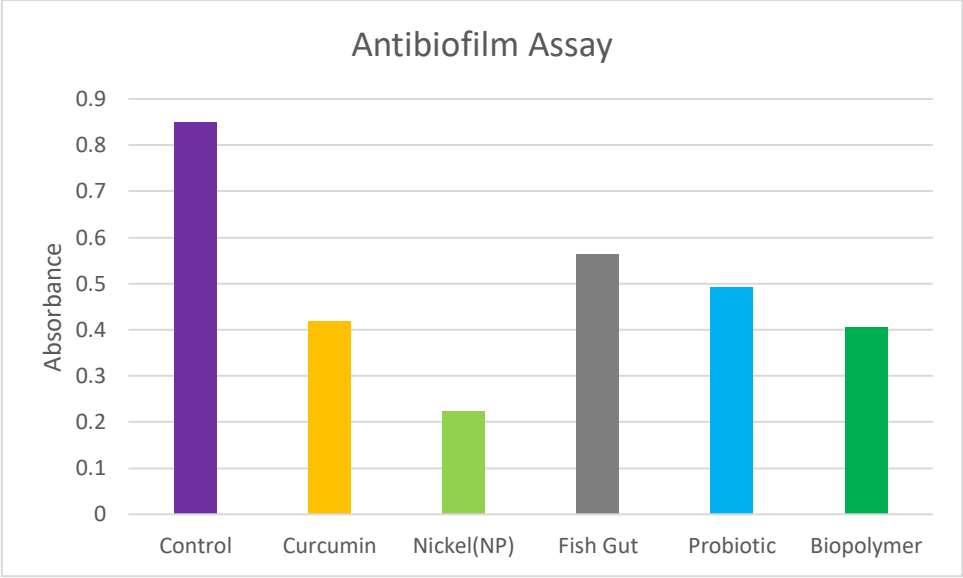

Fig: 12.4 – Absorbance graph of Anti-Biofilm Assay

- **MTT Assay**

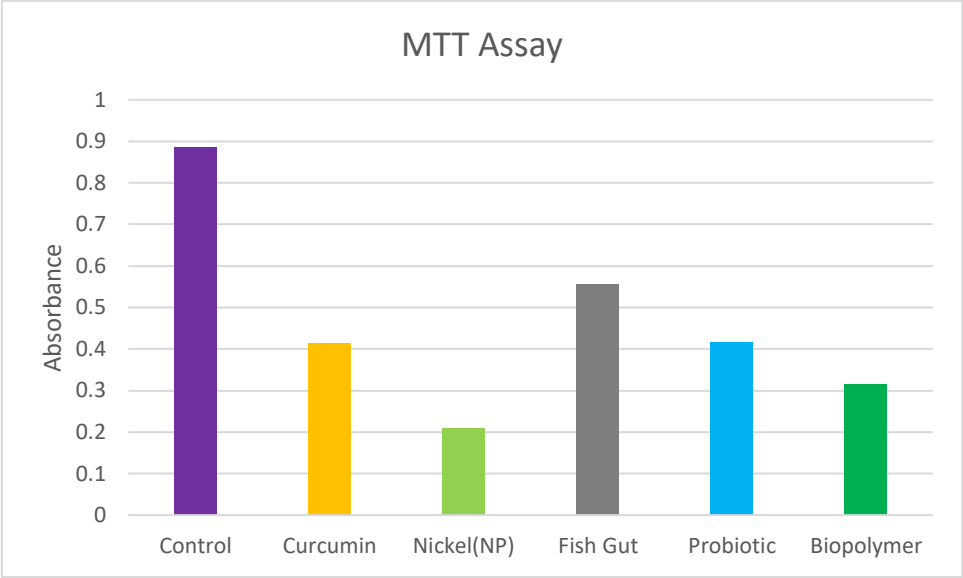

Fig:12.5- Absorbance graph of MTT Assay

- **MIC Assay**

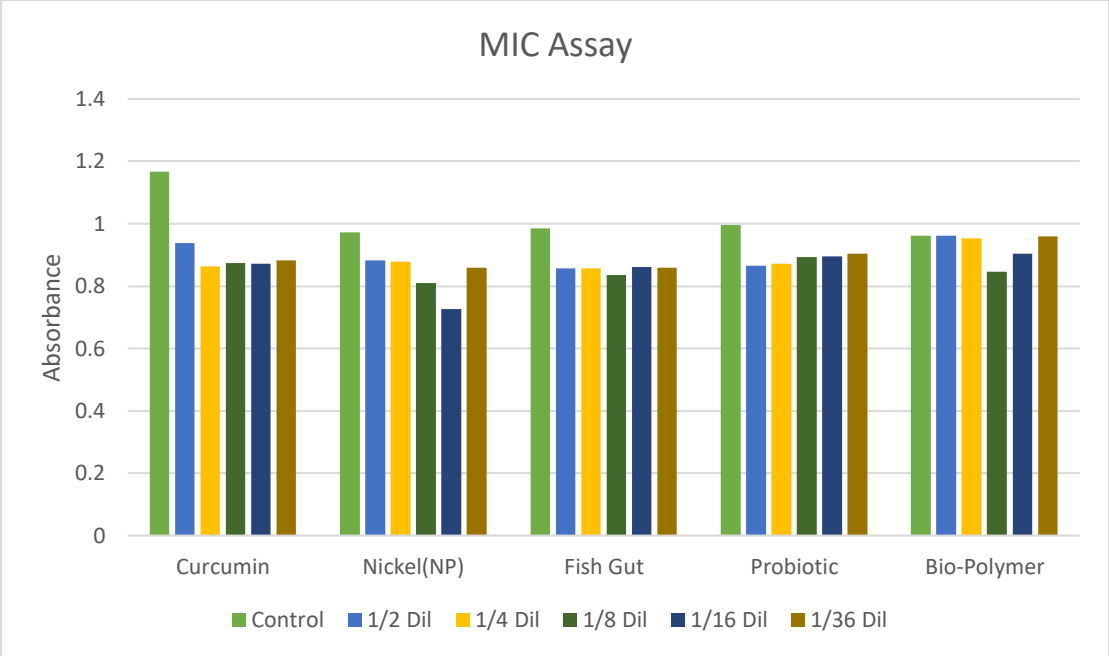

Fig: 12.6 – Absorbance graph of MIC Assay

- **Extraction of Genomic DNA**

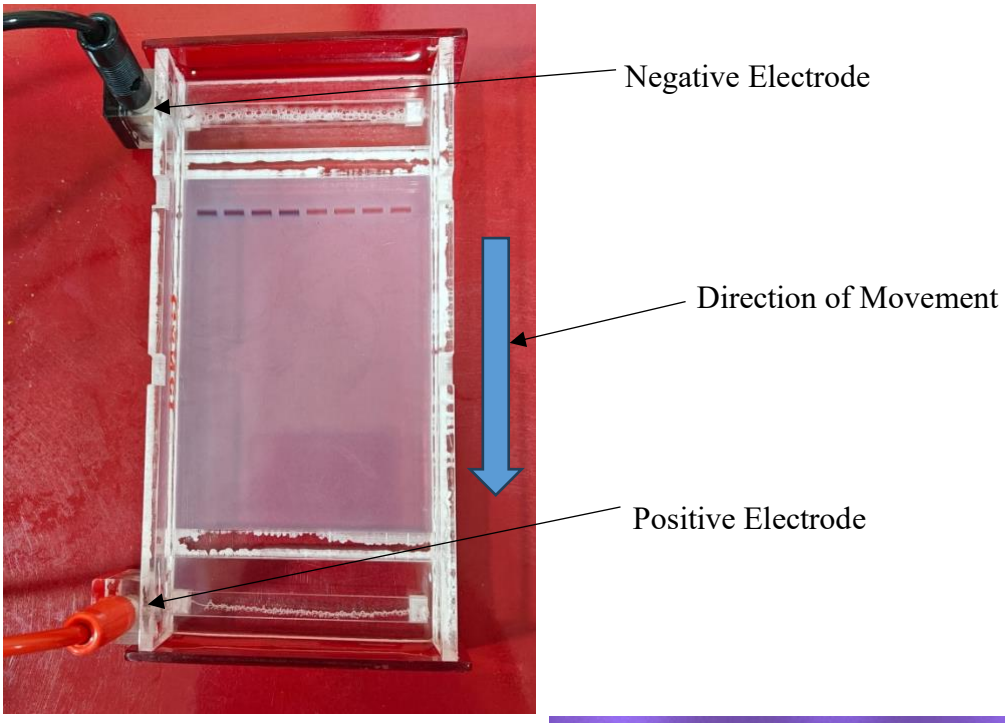

Fig: 13.1 – Agarose Gel Electrophorosis

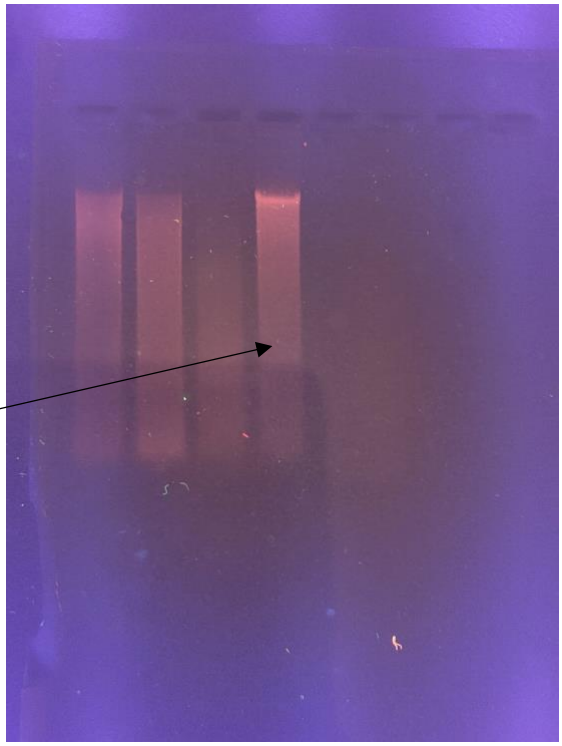

Fig: 13.2 – Genomic DNA Extraction under UV Light

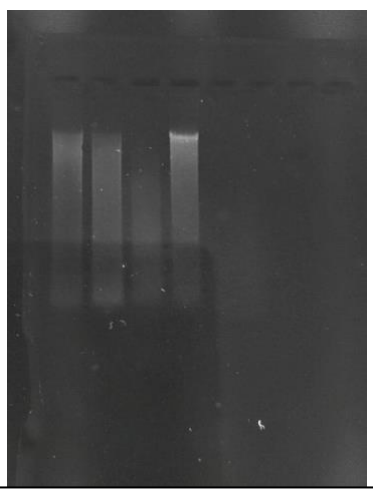

Fig: 13.3 – Genomic DNA Extraction

- **DNA Damage Study of *Candida spp*.**

Fig: 14.1 – Polymerase Chain Reaction of DNA

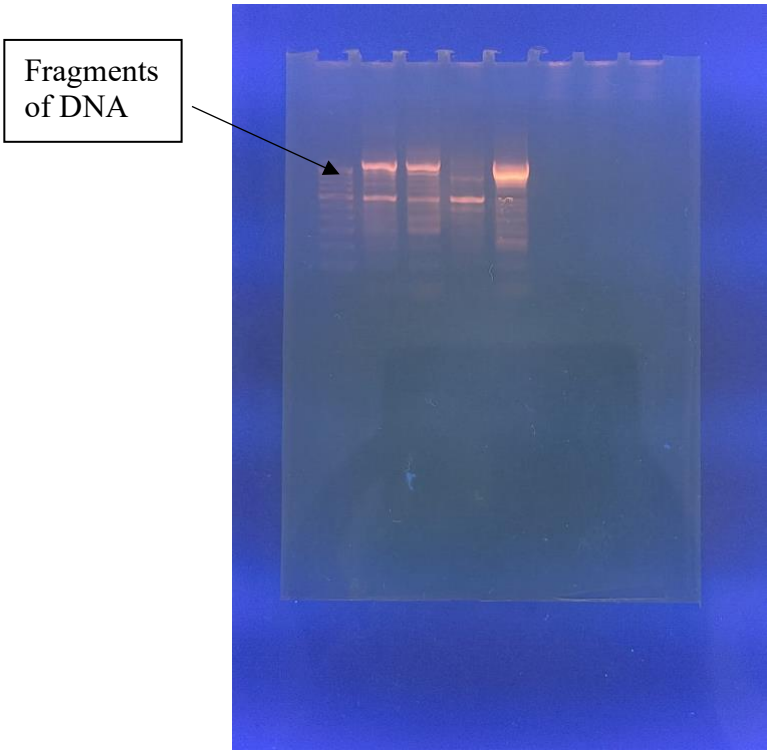

Fig: 14.2 – DNA Damage Study by PCR

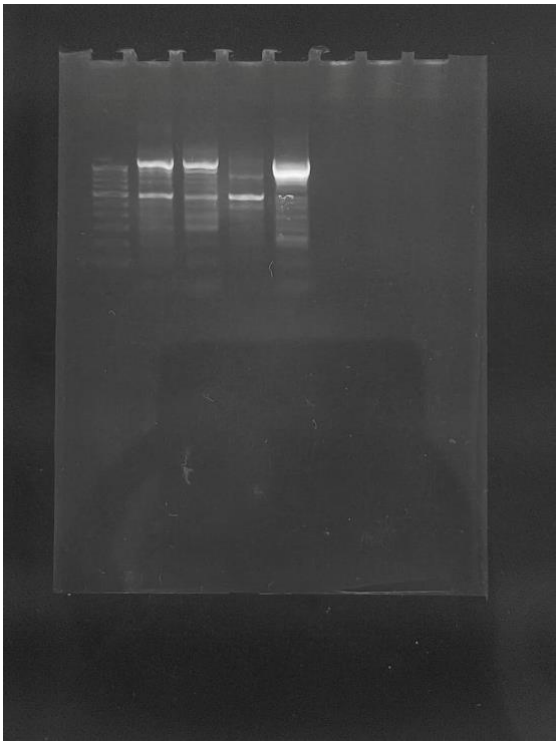

Fig: 14.3 – Migration of DNA Fragments

- **Stress Profiling of Protein Expression**

Fig: 15.1 – SDS PAGE Gel Casting Apparatus

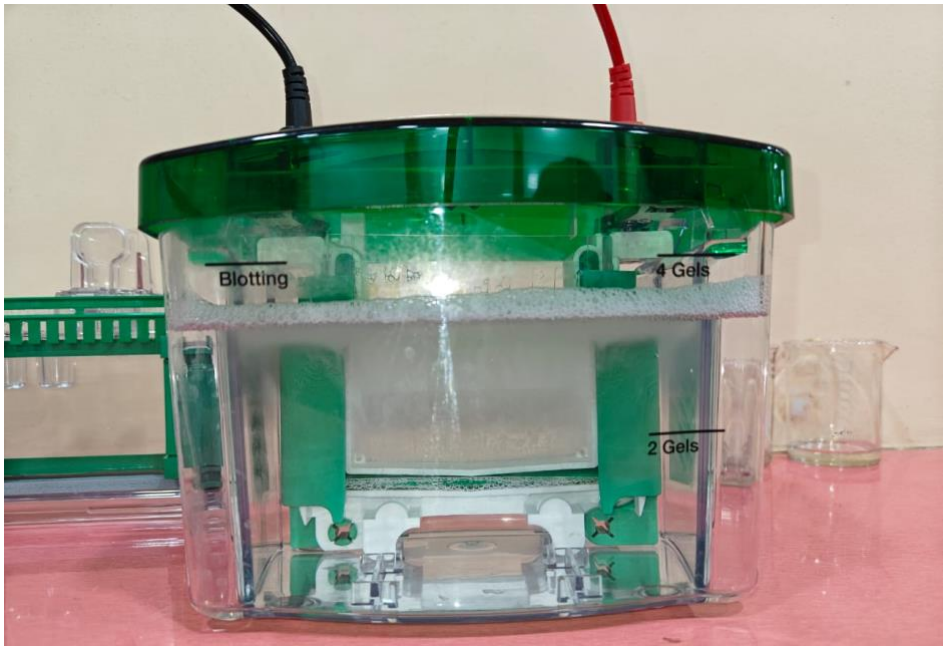

Fig: 15.2 – SDS PAGE Electrophoresis of Protein

- **Stress Profiling of Protein Expression**

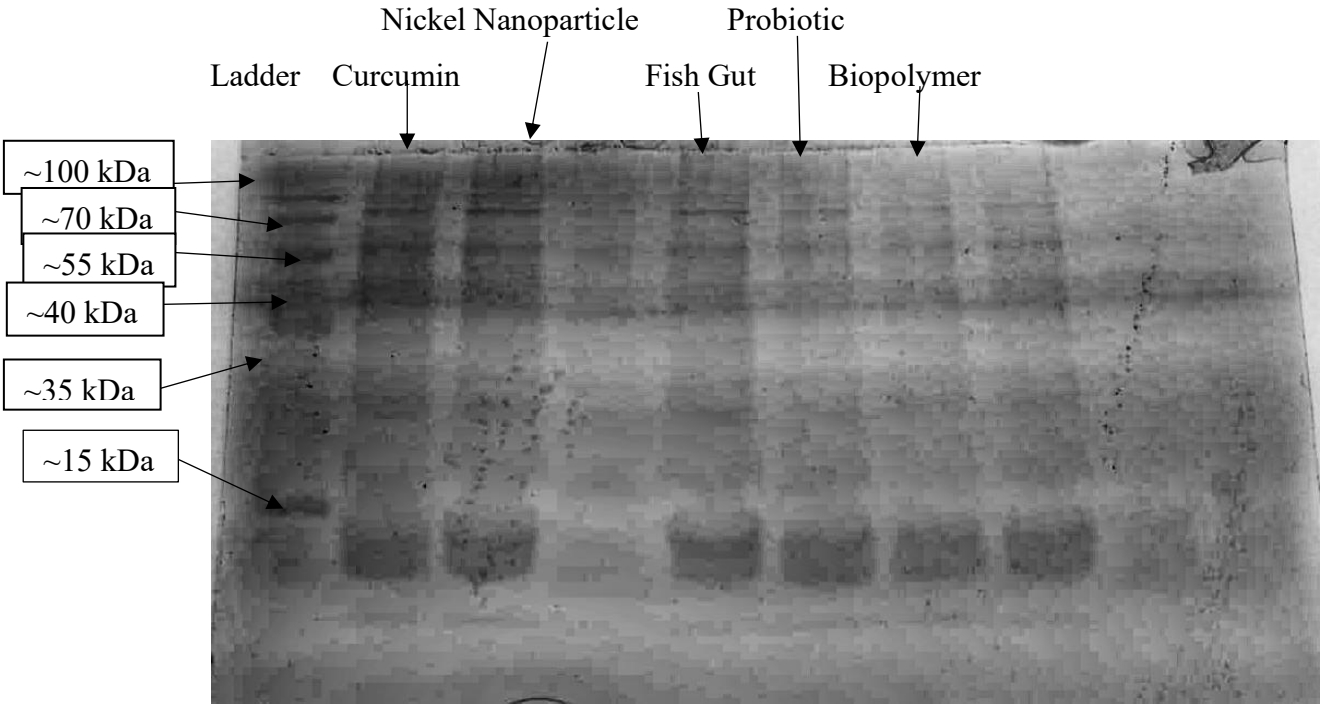

Fig: 15.3 – Stress Profiling of Protein Expression

- **Bioinformatics Documentation of Protein Stress Profiling**

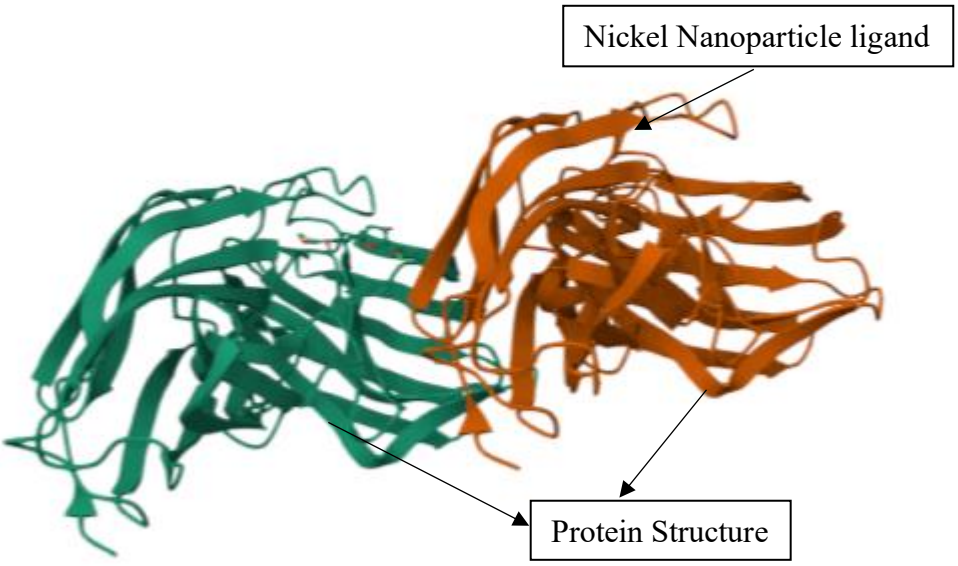

Fig: 16 – Bioinformatics Protein Expression Structure by CB Dock

Protein-Ligand Binding Structure (3D Model) The docking model illustrates a stable interaction between the ligand and the target fungal protein, characterized by compact, well-aligned folding. The close contact between the orange and green domains suggests a favorable binding conformation, supporting the hypothesis that the tested compound—likely curcumin or its nanoparticle conjugate—can interfere with essential protein functions in Candida spp.

Conclusion

The escalating challenge posed by multidrug-resistant *Candida* strains necessitates urgent development of alternative antifungal strategies. This study explored the antifungal and genotoxic potential of biotic and abiotic compounds, including curcumin, nickel nanoparticles, fish gut-derived siderophores, probiotics, and biopolymers. Among these, nickel nanoparticles exhibited the most significant antifungal activity, both in terms of growth inhibition and disruption of biofilm integrity. Curcumin also demonstrated notable efficacy in inhibiting fungal viability and inducing DNA damage (Bai et al. [10]; Rai et al. [2]).

Our findings support the hypothesis that biotic and abiotic agents can exert multifaceted effects on *Candida* spp., not only by compromising cellular integrity but also through molecular and genomic interference. These results position natural and nano-engineered compounds as viable candidates for developing novel, less toxic antifungal formulations (Sharma et al. [1]; Bai et al. [10]).

Nickel nanoparticles synthesized using curcumin as a green reducing agent emerged as the most potent of the five compounds (Kourkoumelis et al. [5]; Rai et al. [2]). Their activity appears to result from multiple mechanisms, including induction of oxidative stress, membrane disruption, and interference with essential metabolic pathways. Importantly, their nanoscale nature may limit the ability of fungal cells to develop resistance rapidly, which enhances their suitability for long-term therapeutic development. Curcumin, though less potent than nanoparticles in some assays, still demonstrated significant inhibitory effects on *Candida* growth and viability. Its known antioxidant and membrane-permeabilizing properties likely contributed to its antifungal action. Moreover, its use in nanoparticle synthesis positions it as both a therapeutic and a functional excipient in formulation science.

The biotic compounds—siderophores and biopolymers—displayed mixed results. Fish gut-derived siderophores showed some antifungal activity, likely due to their iron-chelating properties, which deprive fungal cells of essential nutrients (Verma et al. [7]). However, the probiotic and biopolymer preparations demonstrated minimal direct antifungal effects in vitro. Despite this, their potential role in enhancing host immunity or acting as drug carriers should not be dismissed. Their integration into combinatorial therapies or drug delivery systems may provide additional benefit.

Molecular studies added another layer of insight. PCR-based assays revealed that nickel nanoparticles and curcumin not only inhibit growth but also induce measurable genotoxic effects, as evidenced by altered DNA banding patterns. Additionally, protein expression analysis using SDS-PAGE highlighted the stress-induced responses of *Candida* cells exposed to these treatments, suggesting disruption of protein homeostasis and cellular stress adaptation mechanisms.

This study supports the hypothesis that combining biotic and abiotic strategies may yield effective antifungal outcomes. The use of naturally derived substances like curcumin and microbial metabolites, alongside engineered nanomaterials, provides a multidimensional approach to tackling drug resistance. Such hybrid strategies hold promise not only in inhibiting fungal growth but also in delaying the development of resistance through diverse and overlapping mechanisms of action.

In conclusion, this research contributes to the growing body of evidence supporting the development of novel antifungal strategies rooted in natural products and nanotechnology. While nickel nanoparticles and curcumin emerged as strong candidates for further pharmaceutical development, additional work is needed to explore the full therapeutic potential of other compounds tested. Future studies should focus on in vivo efficacy, cytotoxicity in human cell lines, synergistic effects with existing antifungals, and the molecular pathways underlying their antifungal activity. Ultimately, this integrative approach offers a promising direction in the ongoing fight against MDR fungal infections.

Appendix

Appendix A – Materials and Instruments Used

- **Glassware & Plasticware:**
  - Test tubes, Petri dishes, beakers, measuring cylinders, conical flasks, Eppendorf tubes, pipette tips, capillary tubes, etc.
- **Instruments:**
  - Autoclave
  - Laminar Air Flow Cabinet
  - Centrifuge
  - Spectrophotometer
  - Gel Electrophoresis Apparatus
  - Incubator
  - ELISA Reader
  - PCR Machine
  - SDS-PAGE Gel Electrophoresis Setup
- **Chemicals:**
  - Methanol, Ethanol, Chloroform, Acetone, SDS, NaCl, TE buffer, Phenol, Ethidium Bromide, Coomassie Brilliant Blue, DMSO, Beta-mercaptoethanol, Ammonium Persulfate, Tris-HCl, etc.
- **Biological Reagents:**
  - Lyticase, Proteinase K, RNAse
  - Nutrient Broth, Sabouraud Dextrose Broth and Agar

Appendix B – Fungal and Experimental Strains

- **Isolated Fungal Strains:**
  - *Candida tropicalis*
  - *Candida krusei*
- **Biological Test Components:**
  - Probiotic: *Lactobacillus rhamnosus* (commercial capsule)
  - Fish gut microbiota (collected from Tilapia)
  - Biopolymer (polyethylene glycol + citric acid)
  - Curcumin extract (from turmeric)
  - Nickel nanoparticles (synthesized with curcumin)

Appendix C – Assays Conducted

1. **Cup Plate Assay** – Tested 5 experimental agents for antifungal activity.
2. **MTT Assay** – Evaluated cell viability post-treatment.
3. **MIC Assay** – Determined minimum inhibitory concentration of each compound.
4. **Biofilm Eradication & Anti-Biofilm Assays** – Measured effects on biofilm formation and removal.
5. **Genomic DNA Extraction** – Visualized DNA integrity post-treatment.
6. **PCR-Based DNA Damage Study** – Analyzed DNA disruption through thermal cycling and gel electrophoresis.
7. **SDS-PAGE** – Investigated stress-induced protein expression patterns.

Appendix D – Graphs and Spectra (Reference)

- UV–Vis Spectra of Nickel Nanoparticles
- Absorbance Graphs (from MTT, MIC, Biofilm Assays)
- Gel Images (Agarose electrophoresis of DNA and SDS-PAGE protein bands)

Appendix E – Calculations and Data Sheets

- RF Value Calculations from Thin Layer Chromatography
- Zone of Inhibition (ZOI) Measurement Table
- MIC Dilution Chart
- Optical Density (OD) Readings from ELISA for all assay results

Appendix F – Ethical Considerations

- Fresh fish gut microbiota was obtained from local vendors with appropriate hygiene precautions.
- No animal or human subjects were harmed or used in vivo for this study.
- All procedures involving potential biohazards were conducted following safety guidelines outlined by the university biosafety committee.

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